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ECTOPIC OVARIAN DECIDUA WITHOUT PREGNANCY*

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The first report of decidual transformation outside of the uterine mucosa was made by Walker,[1] in 1887, who observed it in association with an extra-uterine pregnancy. Schmorl,[2] Hirschberg,[3] Rosenberger,[4] Meyer,[5] Geipel,[6,7] Shaw,[8] and Weller[9] have defined the anatomical extent of extra-uterine decidual formations and have discussed their nature and significance, especially in regard to endometriosis. Taussig[10] coined the term ectopic decidua to describe the phenomenon in its extra-mucosal locations, and ectopic decidua (viz., outside of the endometrium) has been noted in association with pregnancy in such diverse locations as the endocervical stroma, the subserosa of the corpus uteri and its ligamentous attachments, the endosalpinx, the serosal surfaces of the fallopian tubes, ovaries, omentum, vermiform appendix, small and large intestine, urinary bladder, and portions of the mesenteries. Meyer[5] stressed the rôle of healed, low-grade, and pre-existing inflammation, especially that associated with the formation of adhesions, in the sensitization of the tissues for the decidual response. Ectopic decidua in pregnancy has been observed in the umbilicus, in laparotomy and hysterotomy scars, and in association with adenomyosis and endometriosis. Weller[9] has pointed out that the regional distribution of the ectopic decidual response corresponds with the distribution of endometriosis. Geipel's[6,7] reports of 40 cases of decidual reactions in pelvic lymph nodes during pregnancy furnish additional evidence of the widespread distribution in the pelvis of tissues that respond to certain stimuli much as does the endometrial stroma. It is the consensus that such ectopic decidual transforma-

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tions occur to some extent in almost every pregnancy after the first trimester.

Decidual transformation in the absence of pregnancy is much less frequent. Under the terms Dysmenorrhoea membranacea and Decidua menstrualis,[11] this phenomenon has been described in the endometrium where it is accompanied by persistent bleeding and pain. Good illustrations of this lesion are presented in the report of Reinhart.[12] Te Linde and Henriksen[13] have furnished additional clinical and pathologic details of such endometrial transformations. Decidual transformation in both the endometrium and the ovary is observed with proliferative lesions of trophoblast, e.g., choriocarcinoma developing after hydatidiform mole or pregnancy, or arising extragenitally without antecedent gestation.

Ectopic decidual reactions in the absence of pregnancy have been reported previously. The report by Schereschewsky[14] of a decidual reaction in an adenomyoma of the rectovaginal septum in a 32-year-old woman must be discounted because of an abortion less than 2 weeks before biopsy. At laparotomy 3 months later for complete extirpation of the lesion, no decidual reaction was visible. The first convincing cases of ectopic decidua unrelated to pregnancy were those of Schiller,[15] who presented 3 unequivocal examples and one which he thought might represent a transitional stage in the development of the cellular response. The 3 clear-cut lesions were all associated with chronic adhesive salpingo-oophoritis. Examination of the excised uteri established the absence of pregnancy. Schiller concluded that ectopic decidua represented a transformation of the pelvic endothelium, i.e., that decidual cells were derived from the peritoneum itself. In a subsequent publication,[16] replying to the criticism of Ulesco-Stroganowa,[16a] who was of the opinion that the ectopic decidual cells were epithelioid and in no way different from the usual type of cell found in inflammatory infiltrates, Schiller added 3 more cases in which ectopic decidua was found on the surface of the ovary and in which the possibility of pregnancy was excluded by gross and microscopic examinations of the uterus.

Other examples of ectopic decidua in the absence of pregnancy have been described by Wallart.[17,18] In contrast to Schiller's observations of surgical material, Wallart's 7 examples were found at necropsy of postmenopausal women ranging from 56 to 88 years in age, 5 being over 70 years. Wallart noted decidual cells not only on the surface of the ovary but also in the loose, cellular centers of corpora albicantia. He accounted for the development of decidua without preg-

nancy in terms of complex neural and hormonal interrelationships among the pituitary body, breast, and ovary in the climacteric.

Shaw[8] found 8 examples of ectopic ovarian decidua in the absence of pregnancy in 33 cases in which oophorectomy had been done. The decidual reaction was observed in the premenstrual phase of the endometrial cycle.

Materials and Methods

Fourteen of the 16 cases presented in this report were received at the Armed Forces Institute of Pathology from federal sources. (One case was contributed by Dr. Frank W. Konzelmann, Emergency Hospital, Washington, D.C., and one by Dr. Emory D. Warner, State University of Iowa.) No special factors are known to have operated in their selection since the decidual reactions were noted as incidental findings in material contributed to the Armed Forces Institute of Pathology for other purposes. The material available for study consisted of the original slides as well as duplicates made from multiple paraffin blocks, formalin-fixed tissue in most of the cases, and clinical and descriptive data. Sections stained with hematoxylin and eosin were used for routine study. The only special methods used were silver impregnations for reticulum and McManus' periodic acid Schiff reagent with and without diastase digestion.

Report of Cases

Case 1 (A.F.I.P. Acc. 92225)

In 1943, a 36-year-old colored woman, gravida ii, para ii, underwent supracervical hysterectomy following a cervical biopsy which was interpreted as squamous cell carcinoma, grade I. No postoperative irradiation was given. Re-examination of the material during the course of this study revealed squamous cell carcinoma *in situ*. Nine years later (1952) recurrent vaginal bleeding prompted another biopsy of the cervix. Adenocarcinoma was found in the cervical stump. The patient was treated with 4,000 r. of x-ray radiation (skin dose) to the pelvis anteriorly and posteriorly as well as with 3,500 mg. hr. of radium by Ernst applicator. Three months later the cervical stump with its tumor, both fallopian tubes and ovaries, and numerous pelvic lymph nodes were excised.

No distinctive features were recognized in the examination of the excised tissue. Most of the anatomical landmarks were obliterated by the preceding irradiation. In the fixed state the ovaries and tubes were adherent to each other and were firm, shrunken pieces of tissue, each measuring less than 3 cm. in greatest dimension.

Microscopic examination showed both ovaries to be composed of dense fibrous tissue with many corpora albicantia. Patches of fibrotic ovarian stroma were recognizable. On the surface of each ovary there was patchy loosening of the stroma, and in some of these areas, rounded nests of decidual cells were seen. This alteration of the stroma was found both at free margins and underlying dense fibrous tubo-ovarian adhesions. The decidual cells were polygonal, contained a small, often eccentric nucleus surrounded by a slightly basophilic cytoplasm, and were loosely arranged in a myxoid matrix. In one section an adrenal cortical rest was found in the meso-

varium. In another section a round accumulation of hilar cells was identified. No corpus luteum was found in sections of either ovary.

Case 2 (A.F.I.P. Acc. 216150)

A 49-year-old woman, gravida ii, para i, abortus i(?), had complained of uterine bleeding of about 6 months' duration. She was treated with x-ray radiation of unstated amount without success. A presumptive clinical diagnosis of submucous leiomyoma of the uterus was made, and hysterectomy and bilateral salpingo-oophorectomy were done. It is inferred from the record that about 4 months elapsed between the last x-ray treatment and the operation.

The uterus contained a submucous leiomyoma approximately 4 cm. in diameter. No products of conception were seen. The ovaries were small, firm, and shrunken, measuring no more than 2.5 cm. in greatest dimension.

Most of the ovary was composed of corpora albicantia and medullary tissue. The cortex was thin and somewhat fibrotic. Along the free margin a few patches of loosened stroma, composed of polygonal decidual cells with clearly defined cell interfaces, were seen just below the surface in the cortex. Underlying this area of focal decidual transformation were the remains of a corpus luteum, with disorganized architecture and degenerating cells.

Case 3 (A.F.I.P. Acc. 260099)

A 51-year-old white woman in active menstrual life had complained of menometrorrhagia of 2 to 3 months' duration. Curettage of the endometrial cavity revealed a well differentiated adenocarcinoma. She was treated with 3,600 mg. hr. of radium inserted into the uterine cavity. Seven weeks after irradiation hysterectomy and bilateral salpingo-oophorectomy were performed.

Examination of the uterus revealed a small tumor covered by a membranous slough. The tumor was superficial and did not invade the myometrium deeply. No products of conception were found in the uterus. The fallopian tubes and left ovary were without notable gross lesions. A papillary projection less than 1 cm. in diameter was observed on the surface of the right ovary at the lateral pole.

Microscopic examination of the papillary structure in the right ovary showed that it was a serous papilloma. However, the lateral pole of the left ovary contained a central area composed of a few cystic spaces of small to medium size surrounded and traversed by a transformed stroma. The cystic spaces were lined by a single layer of columnar epithelial cells which resembled endometrial epithelium; an occasional length of epithelium contained vacuolated cells. The stroma of this area was composed of compactly arranged polyhedral decidual cells, with clear, faintly basophilic cytoplasm and well demarcated acidophilic cell boundaries. This decidual stroma extended between the cysts, and the lining of the cysts in some places rested upon such stroma. A few nests of decidual cells were found in the cortex a short distance from the cystic area. This lesion was interpreted as endometriosis with decidual transformation of the stromal component. Subjacent to this lesion, toward the medullary portion of the ovary, was a somewhat shrunken and disrupted corpus luteum.

Case 4 (A.F.I.P. Acc. 282199)

A cystic mass in the region of the right ovary was found on routine examination of a 43-year-old woman. At laparotomy, a cyst was removed from the right ovary and a biopsy was performed on a nubbin of tissue on the surface of the left ovary. The cervix was coned, the endometrial cavity curetted, and an appendectomy was done.

A mild degree of chronic cervicitis was found. The curettings showed the endometrium to be in the early secretory phase. No products of conception or evidence of recent pregnancy were found. The cyst in the right ovary was thin-walled, measured 8.5 cm. in diameter, and was a pseudomucinous cystoma. The appendix was fibrosed. The nubbin of tissue from the left ovary measured 0.7 by 0.5 cm., was composed of firm whitish tissue, and had a tiny cystic area, 0.2 by 0.1 cm., at its surface.

Microscopic examination of this fragment showed that most of it was normal ovarian cortex. The small cyst was lined by a layer of columnar epithelial cells resembling endometrial glandular epithelium or altered peritoneum of an inclusion cyst. Similar, smaller cystic spaces were found nearby. Adjacent to this area were a few delicate adhesions that formed a pannus-like coating over the ovarian surface. These adhesions were relatively free from inflammatory infiltration, only a few lymphocytes and plasma cells being present. The stroma of the adhesions was composed of polyhedral decidual cells which extended for a short distance into the ovarian stroma. Most of the decidual cells were loosely arranged and had indistinct cell borders, but in some foci they were closely packed and the cell borders were distinct. The surfaces of the adhesions were covered by a single layer of flat mesothelial cells, and the decidual cells lay subjacent to this single cell layer. The presence or absence of a corpus luteum in this ovary could not be determined from the data at hand. No corpus luteum was found in the opposite ovary.

Case 5 (A.F.I.P. Acc. 300087)

A 39-year-old Army nurse, gravida 0, had complained of menometrorrhagia since the removal of cervical polyps 3 years previously. Physical examination revealed an enlarged, nodular uterus. A hysterogram was interpreted as showing a submucous leiomyoma. At laparotomy both ovaries were adherent to the parietes, and "chocolate" cysts were found bilaterally. The entire uterus and adnexal masses from both sides were removed.

The uterus was small. The endometrium was smooth and uniform. A submucous nodule was present. Both ovarian masses and fallopian tubes were covered with rough, coarse adhesions. A cystic corpus luteum was recognized in the left ovary. Both ovaries were distorted considerably by cysts of small and medium size, some filled with chocolate material, others with clear fluid.

Microscopic examination showed the endometrium to be slightly hyperplastic and the submucosal nodule to be a leiomyoma. Both ovaries were diffusely involved by cystic endometriosis with old and recent hemorrhage, and scarring. The cystic corpus luteum in the left ovary displayed no unusual features. The right ovary contained a few regressing corpora lutea and corpora fibrosa, none of recent vintage. Three of these structures contained decidual cells. In one instance the decidual transformation was in the form of a radial band from the center toward the periphery of the corpus luteum; in the other two, it was in the form of a discrete patch of altered stroma. There was no evidence of decidua near the peritoneal surface in any of numerous sections. The decidual cells were clearly distinguishable from the adjacent regressing luteal cells.

Case 6 (A.F.I.P. Acc. 339991)

A 49-year-old white woman, gravida i, para i, underwent hysterectomy and bilateral salpingo-oophorectomy because of symptoms related to the menopause, including disturbed menstrual flow. There was severe cystic cervicitis. No products of conception or evidence of recent pregnancy were found in the endometrium. A few leiomyomas were seen in the myometrium. Chronic adhesive salpingo-oophoritis was noted.

Microscopic examination revealed a large corpus luteum in one ovary (side not stated). The surface of this ovary was covered by many fine and coarse adhesions with varying numbers of lymphocytes and plasma cells. In many areas these adhesions covered the ovarian surface like a pannus, producing entrapped lacunae lined by intact peritoneal mesothelium. Within these subperitoneal adhesions, many aggregates of decidual cells were found. These varied from clusters of a few cells to patches more than one low-power field in extent. On occasion, the decidual transformation extended into the cortex of the ovary. The decidual cells were morphologically characteristic and displayed some variety in form, arrangement, and tinctorial reaction. In some areas they were loosely arranged in an edematous, myxoid matrix; in other areas the cells were in close apposition, with acidophilic boundaries sharply delimiting individual cells. The cytoplasm of some of the cells was strikingly opalescent. In the absence of decidual transformation, the cortex of the ovary was thick and cellular.

Case 7 (A.F.I.P. Acc. 538037)

A 35-year-old woman, gravida 0, para 0, had complained of postmenstrual vaginal discharge. Biopsy of the cervix revealed infiltrative squamous cell carcinoma. She was treated with 7,740 mg. hr. of radium and 8,000 r. of x-ray radiation (skin dose) to the pelvis anteriorly and posteriorly. Hysterectomy, bilateral salpingo-oophorectomy, and pelvic lymph node dissection were performed 10 weeks after radiation therapy was completed.

The uterus measured 7 by 4 cm. Residual tumor was seen in the cervical canal. No products of conception were found in the endometrium. Two leiomyomas were present in the myometrium. The right fallopian tube was dilated and contained purulent material. The right ovary measured 2.5 by 2 cm., and was smooth, glistening, and appeared to be replaced by a firm, white, friable tissue. The left tube and ovary were similar in appearance to the right.

Microscopic examination revealed residual squamous cell carcinoma in the cervix. The endometrium was in a proliferative phase. The fallopian tubes showed acute follicular salpingitis and pyosalpinx. On and below the peritoneal surface of one ovary and above a disorganized corpus luteum, a few small patches of decidual cells were loosely arranged in an edematous matrix. Additional blocks from both ovaries failed to reveal any more decidua.

Case 8 (A.F.I.P. Acc. 546894)

A 27-year-old white woman, gravida 0, para 0, had complained of irregular vaginal spotting. Biopsy of the cervix revealed infiltrating squamous cell carcinoma. She was treated with 7,740 mg. hr. of radium and 7,200 r. of x-ray radiation to the pelvis anteriorly and posteriorly. Hysterectomy, bilateral salpingo-oophorectomy, and pelvic lymph node dissection were done 12 weeks after irradiation therapy was completed.

The uterus measured 7.5 by 4 by 1.5 cm. The cervix was covered by smooth epithelium. The endometrial cavity was smooth and regular. No products of conception were identified. The ovaries were small, but normal in contour.

Microscopic examination failed to reveal any residual squamous cell carcinoma. The endometrium was in a proliferative phase. There was a diffuse radiation effect throughout the fallopian tubes, uterus, and ovaries. A cystic corpus luteum was present in one ovary, compressing the overlying cortex. On the peritoneal surface near this structure a fairly extensive decidual transformation which extended into the cortex was associated with a minimal inflammatory reaction consisting of scattered lymphocytes and a few delicate fibrous tags at the surface. On the peritoneal

surface of the other ovary there was a less conspicuous decidual change not associated with inflammation. A similar reaction was found on the surface of the mesosalpinx in an area of fibrosis with focal psammoma body formation.

Case 9 (A.F.I.P. Acc. 555513)

A 33-year-old white woman, gravida v, para iv, had complained of pain in the right side associated with menometrorrhagia of 7 months' duration. Examination revealed a firm, fixed mass, 5 cm. in diameter, in the right adnexal area. The uterus, both fallopian tubes, and left ovary were described in the surgeon's note as normal. A right salpingo-oophorectomy was performed.

The fallopian tube was kinked and tortuous. The ovarian tissue measured 5.5 by 3.5 cm. and was densely adherent to the tube. It was composed of a fairly well demarcated mass of yellowish gray tissue surrounded by a zone of recognizable ovarian cortex in which a corpus luteum was identified.

The mass within the ovary consisted of a dense mixture of lymphocytes, plasma cells, and histiocytes with occasional eosinophils and polymorphonuclear leukocytes filling a large abscess cavity. The inflammatory lesion involved chiefly the medulla, but patches of exudate and edema disrupted the cortex. The peritoneal surface was covered with coarse adhesions. In only one of many sections did a few of these adhesions contain small nests of decidual cells with sharp borders. A small corpus luteum was disrupted by the diffuse inflammatory reaction. The fallopian tube exhibited subacute adhesive salpingo-oophoritis.

Case 10 (A.F.I.P. Acc. 564300)

A 65-year-old woman, gravida ii, para i, had complained of painless vaginal bleeding of 2 years' duration. The uterus was enlarged to twice normal size. Curettage revealed a fairly well differentiated adenocarcinoma of the endometrium. A hysterectomy and bilateral salpingo-oophorectomy were performed 9 days later. There was no mention of any radiation therapy before operation.

The uterus measured 11.5 by 7.2 by 6.5 cm. The endometrial cavity contained a large friable mass of yellowish and reddish tissue. The fallopian tubes were not remarkable. Both ovaries were small and firm, the left measuring 2.7 by 1.2 by 0.8 cm.; the right, 1.4 by 1.1 by 0.8 cm. Numerous corpora albicantia and a white nodule measuring 0.6 by 0.5 by 0.3 cm. on the surface of the right ovary were noted.

Microscopic examination confirmed the presence of an endometrial adenocarcinoma which invaded the myometrium. An area of cortical fibrosis corresponding to the small nodule seen grossly and a few delicate fibrous adhesions free from inflammatory cells were seen in the right ovary. The central portion of both ovaries was occupied by large and small corpora albicantia. In the cortex near the surface of the left ovary above the corpora albicantia there was a zone of decidual cells which were irregularly dispersed, and associated with a scanty infiltration of lymphocytes and an occasional eosinophil. No corpus luteum was identified in the vicinity. A few delicate fibrous adhesions were present nearby, but these did not contain decidual cells.

Case 11 (A.F.I.P. Acc. 574297)

A 40-year-old white woman, who had had a radical mastectomy 1 month previously for carcinoma of the breast with lymph node metastases, was treated further by hysterectomy, bilateral salpingo-oophorectomy, and appendectomy.

The uterus measured 6 by 4 by 3 cm. No gross abnormalities of the uterus, fallopian tubes, ovaries, or appendix were observed. No products of conception were found in the endometrium, which was in a proliferative phase. Both ovaries showed

distortion and scarring secondary to diffuse endometriosis and healed adhesive perioophoritis. They contained several small cysts, lined in part by endometrium-like epithelium and in part by endometrial stroma-like tissue admixed with granulation tissue and hemosiderin-laden macrophages. No fresh corpora lutea were found in either ovary, but numerous corpora fibrosa and albicantia were present in the medullae. The overlying cortices were thinned and scarred. In the medulla of one ovary immediately beneath a cortical scar was a corpus albicans with a loose and fibrous center which contained many hemosiderin-laden macrophages. At the margin of this corpus albicans was a single patch of compactly arranged decidual cells with polygonal outlines, pale basophilic cytoplasm, vesicular nuclei, and sharply defined cell borders. This patch was made up of less than 40 decidual cells and a few lymphocytes and it could not be detected in further sections prepared from the original paraffin block. In multiple sections from this ovary another patch of decidual cells, slightly smaller than the first, was found adjacent to a corpus albicans near the corticomedullary junction.

Case 12 (A.F.I.P. Acc. 512572)

A 42-year-old white woman had complained of severe menorrhagia and dysmenorrhea of 6 months' duration. Hysterectomy and right salpingo-oophorectomy were performed. The uterus measured 8.5 by 5.5 by 4 cm. and contained several small, spherical leiomyomas. The endometrial cavity was lined by smooth epithelium; no products of conception were identified. The right fallopian tube was not remarkable. The right ovary measured 4 by 3 by 2 cm. and contained several corpora lutea of varying ages, including a recent one with a central cavity filled with gelatinous material.

Microscopic examination showed the uterus to be lined by endometrium in an early secretory phase. The tumors in the myometrium proved to be leiomyomas. No lesions were present in the fallopian tube. Arising from the surface of the ovary above a large corpus luteum and separated from it by a layer of compressed ovarian cortex was a single, delicate, fibrous adhesion packed with swollen, polyhedral decidual cells. The nuclei were small, often eccentric, and relatively clear. The cytoplasm was slightly basophilic and the cell borders sharply demarcated. In several blocks taken from this ovary only a rare, delicate, fibrous adhesion was encountered at the surface. In one of these, also not far distant from the corpus luteum, a few decidual cells were identified.

*Case 13 (A.F.I.P. Acc. 582760)**

A 48-year-old woman, gravida 0, had complained of severe menorrhagia of over 1 year's duration. Ten years previously a right salpingo-oophorectomy had been performed. No history of endocrine therapy was elicited. At laparotomy the right fallopian tube and ovary were absent; the left fallopian tube also was absent. The omentum, small bowel, colon, and uterus were bound together in a mass of adhesions. The uterus, left ovary, and a portion of omentum were removed.

The uterus measured 5.5 by 5 by 3.5 cm. Its serosal surface was diffusely roughened and numerous shaggy adhesions were present. The myometrium contained a small leiomyoma. The endometrium was smooth and thin; no products of conception were present. The left ovary measured 3 by 2.5 by 1.5 cm., and a few adhesions were seen at its surface. Also noted on the surface were several irregularly dispersed, small, whitish elevations less than 1 mm. in diameter. At one pole of the ovary there was a corpus luteum about 0.6 cm. in diameter.

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* Contributed by Dr. Frank W. Konzelmann, Emergency Hospital, Washington, D.C.

The endometrium was in a proliferative phase. Diffuse, chronic perimetrosalpingo-oophoritis was present. Beneath the peritoneal surface and extending into the substance of the ovarian cortex were many clusters of cells which were characteristic of decidua. These cells tended to be arranged in spherical nests but also had diffused into the cortex. The decidual reaction was greatest near the corpus luteum, but several nests of decidua were present in sections some distance from this structure. Many of the foci of decidual reaction were associated with peritoneal adhesions. The decidual response in this case was more extensive than in any other of this series.

*Case 14 (A.F.I.P. Acc. 580367)**

A 44-year-old woman, multigravida, had complained of irregular vaginal bleeding for 2 years. A curettage was performed and a diagnosis of adenocarcinoma of the endometrium was made. The patient received 4,800 mg. hr. of radiation. One month later, panhysterectomy and bilateral salpingo-oophorectomy were performed. The pertinent diagnoses were adenocarcinoma of endometrium, with radiation changes, and pseudomucinous cystadenoma of the ovary. In the opposite ovary, a regressing corpus luteum was found. Adjacent to this on the ovarian surface, a microscopic focus of about 20 typical decidual cells was seen. Another nodular collection of similar cells lay immediately beneath the ovarian surface adjacent to a small zone of hemorrhagic peri-oophoritis.

*Case 15 (A.F.I.P. Acc. 582924)**

A 42-year-old woman, gravida i, para i, had complained of menometrorrhagia for 9 months. Curettage of the endometrium revealed adenocarcinoma. Panhysterectomy and bilateral salpingo-oophorectomy were done 7 weeks after intracavitary irradiation. Sections of the uterus contained areas of adenocarcinoma of the endometrium with irradiation changes. One ovary contained a cystic corpus luteum. On and just beneath the ovarian surface, three minute foci of well preserved decidual cells with loose myxoid stroma were noted. They were covered by a thin inflammatory membrane containing a few lymphocytes. A small nodule of typical interstitial cells was seen in the hilus of the ovary.

Case 16 (A.F.I.P. Acc. 604713)†

A 64-year-old nulliparous white widow was admitted with cardiac failure of moderate severity. Menopause had occurred 15 years previously. A large cystic mass in the region of the left ovary extended out of the pelvis almost to the umbilicus. The patient admitted having been aware of this mass for only 2 or 3 months. At laparotomy a loculated cyst involving the left ovary was found and approximately 1,200 cc. of clear fluid were evacuated. The uterus was small and knobby, with numerous leiomyomas. A "chocolate cyst" involved the right broad ligament and right ovary.

The lining of the left ovarian cyst was smooth and shiny and showed no adenomatous areas. Microscopic examination revealed leiomyomas and adenomyosis of the corpus uteri and endometriosis of the ovaries. A few scattered foci composed of large, pale-staining cells typical of decidual tissues were found in the ovaries but were not associated with the areas of endometriosis.

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SUMMARY OF PATHOLOGIC CHANGES

The ectopic decidual reaction was located immediately subjacent to the peritoneal surface and in close proximity to a recent corpus luteum (frequently disrupted) in 8 cases (cases 2, 3, 6, 7, 8, 9, 12, and 13). In case 1, no corpus luteum was found; an adrenal rest was present in the mesovarium and a nest of hilar cells was found in the medulla of the ovary. A similar hilar cell nest was present in case 15. In case 4, the presence or absence of a corpus luteum could not be determined. In case 5, the decidual reaction was in a regressing corpus luteum and also in corpora fibrosa and albicantia; a recent corpus luteum was present in the opposite ovary. In case 9, the decidual reaction consisted of one small focus of transformed cells some distance from the corpus luteum. In case 11, the decidual reaction also consisted of two small foci of cells, each adjacent to a corpus albicans in which no functioning luteal cells were identified. In case 12, the decidual reaction was limited to two small peritoneal adhesions near a normally formed, fresh corpus luteum.

Periovarian adhesions of a postinflammatory nature were present to a significant extent near the ectopic decidual reaction in 14 of the 16 cases, and the reaction was located both in the adhesions and in the cortex just below them. A history of recent radium or x-ray treatment to pelvic structures was given in 9 of the 16 cases. In 5 cases the decidual reaction was associated with endometriosis; in one of these there was a history of previous irradiation.

Cases 10 and 16 are exceptional in this series in that there was no corpus luteum, no previous irradiation, and no significant inflammatory adhesions. In case 16 an old "chocolate" cyst, presumably endometrial, was found in the ovary. These 2 cases closely resemble those reported by Wallart.[17,18]

The uterus was available for examination in 15 of the 16 cases. No evidence of pregnancy or of recently terminated pregnancy was found in any of them. In case 9, the uterus was not available for study, but was reported as normal in the surgeon's notes. There is nothing in the antecedent or subsequent history of this patient to suggest pregnancy.

The identification of cells as decidual rests upon their resemblance to the decidual cells seen in the endometrium during the late progestational stage of the menstrual cycle and in pregnancy. Weller's[9] description of ectopic decidual cells is an adequate standard: "nests of large cells with slightly basophilic cytoplasm and spherical, usually

eccentric, nuclei, ... often 30 microns or more in diameter." To this description might be added the generally polygonal shape of the cells, their tendency to form compact pavement-like tissue simulating the stratum compactum or, alternatively, a looser meshwork simulating the stratum spongiosum of the decidua vera, and the frequent refractile acidophilia of the intercellular substance. The cells observed in the ovary in these 16 cases satisfy these morphologic criteria.

Geipel[7] pointed out that decidual cells, whether in the endometrium or in ectopic location, contain glycogen. In 14 of the 16 cases in this series, material was available for treatment with the periodic acid-Schiff (PAS) method. In each of these 14 cases Schiff-positive material was found both as an intensely colored zone of fuchsinophilic material condensed at the peripheral portion of the cells in question and as inconstant smaller particles in the relatively clearer perinuclear zone. Parallel sections digested with diastase showed that this material was not completely digested. Although the particles in the perinuclear areas were blanched, traces of the fuchsinophilic material remained at the cell border. The most striking illustration of this partial digestion was in case 13. Whether this observation indicates the presence of small amounts of mucopolysaccharide along with the larger moiety of glycogen cannot be determined. Shaw[8] stressed the value of demonstrating argyrophilic intercellular fibers between decidual cells to distinguish them from swollen cells which might simulate decidua. In the same 14 cases, sections treated by Gridley's reticulum method showed delicate, often intertwined, intercellular fibrils upon which silver had been deposited.

The identification of the two patches of decidua in case 11, and the decidua in the two delicate peritoneal adhesions in case 12, was made by comparison with the appearance of similar cells found in the other 14 cases in routine hematoxylin and eosin preparations. The quantity of decidua in these two cases was not sufficient to permit the use of periodic acid and silver impregnation techniques. In case 16, only sections stained with hematoxylin and eosin were available for study.

Discussion

The cells which are transformed into decidual cells in response to appropriate stimuli are stromal in origin and derived from the subcelomic mesenchyme, the same embryonic tissue from which the endometrial stroma develops. In the ovary this tissue is represented by an inconspicuous layer of cells directly beneath the peritoneal surface which blends imperceptibly with the ovarian cortex. This

accounts for both the gross appearance of the lesions, "small nodules, streaks and patches produced by a thickening of the serosa or, more properly, the subserosa" (Weller[9]), and the microscopic observations described above. In the early embryo, this subcelomic mesenchyme surrounds both the wolffian and müllerian ducts in the urogenital ridge. The two ducts are in close proximity at the time of their formation as evaginations from the celomic lining, and it is not surprising that occasionally a kidney removed during pregnancy should display a decidual reaction in the renal pelvis, as described by Bettinger.[19] The capacity to be transformed into decidua under appropriate conditions is an inherent and unique property of the mesenchyme of the urogenital ridge and its derivatives.

The conditions under which, and the mechanisms by which, this transformation occurs are generally constant, but can vary within certain limits. Pregnancy is the usual condition in which decidua develops, either in the endometrium or ectopically, presumably as the response to progesterone elaborated by the corpus luteum and the syncytiotrophoblast. However, decidua can develop physiologically in the absence of pregnancy, i.e., without trophoblast. The predecidua in the endometrium from the 23rd through the 28th day of the hypothetically normal menstrual cycle is cytologically indistinguishable from true decidua; the only difference is quantitative. Conversely, decidua may develop both in the endometrium and ectopically in association with lesions of trophoblast, notably choriocarcinoma. In such cases it is presumed that the decidual transformation is not the result of the direct stimulus of the chorionic gonadotropin but is mediated through the intervening stimulus of luteinized theca cysts in the ovary. These respond to the APL substance in the trophoblast by liberating an excessive amount of progesterone, which, in turn, is the stimulus for formation of decidua. The failure to observe decidual reactions beneath the peritoneal surfaces in males with choriocarcinoma, genital or extragenital, is added evidence that the intermediate position of the lutein cell and the progesterone it elaborates is a necessary link in the chain.

Loeb[20] originally produced deciduomas in the uteri of guinea-pigs by non-specific trauma during the lutein phase of the estrous cycle. This reaction occurred only during the active phase of the corpus luteum, and it was clear that the synergistic actions of local trauma and progesterone were the essential conditions for this phenomenon. Subsequent observations by a number of investigators have confirmed Loeb's results. Notable among these are the investigations of Unter-

berger,[21] who produced ectopic deciduomas in the uterine serosa of virgin rabbits by making incisions in the serosa, then mating the rabbit. Goldstein and Tatelbaum[22] were able to produce deciduomas in guinea-pigs by injecting an alcoholic extract of rabbit corpus luteum. The decidual transformations which they produced were both in the endometrium and beneath the uterine serosa following incisional trauma. Recently Stuermer and Stein[23] demonstrated that endometrium grown *in vitro* assumes the fibroblastic form when grown in ordinary media, but assumes an epithelioid shape, much like sheets of decidual cells, when grown in a medium enriched by serum from umbilical cord.

In the present series the ectopic decidua in all but 4 cases (cases 1, 10, 11, and 16) was found in close proximity to either an active corpus luteum anatomically damaged by ionizing irradiation or other inflammatory process, or one in some stage of regression. In those cases in which the ectopic decidua was not at the surface of the ovary or in inflammatory adhesions at the surface (cases 5 and 11), the presence of subcelomic mesenchymal cells is explained by mechanical transportation of these cells during the cycle of follicle rupture, luteinization, and involution, much as peritoneal cells may be drawn into the substance of the ovary. In all but 2 cases, either a recent and active or an old and healed inflammatory reaction involved the ovarian surface. Ionizing irradiation therapy for a variety of gynecologic complaints was often sufficient to traumatize the stromal cells so that they would respond to the hormonal stimulus. Lacassagne, Fehr, and Nyka[24] demonstrated the formation of uterine deciduoma in rabbits following doses of x-ray sufficient to kill ova but insufficient to destroy corpora lutea.

Cases 1, 10, 11, and 16 differ from the other cases in this series in that no corpus luteum, either active or involuting, was present in either ovary. In cases 10 and 16 the patients were chronologically postmenopausal; in case 1, radiation therapy for adenocarcinoma of the cervix had been given more than 3 months previously, and not even disorganized luteal structures remained subjacent to the decidual reaction as in cases 4, 8, and 9. A source for the progesterone or progesterone-like stimulus other than the corpus luteum must be postulated in these cases. The clue furnished by the observation of an adrenal cortical rest in the mesovarium in case 1 implicates the adrenal cortex as a possible source; the hilar cell nest does not seem to be a likely source of progesterone. Engelhart[25] was able to induce progestational changes (secretion and formation of decidua) in the

endometrium of virgin rabbits by injecting a lipidic extract of the human adrenal cortex. Callow and Parkes[26] confirmed and extended the observations of Engelhart to include extracts from the adrenal glands of cattle, horses, pigs, and sheep. Beall[27] isolated progesterone as a chemically pure substance from the adrenal cortex of oxen. However, progesterone is not unique among steroid hormones in its ability to produce progestational effects. McKeown and Zuckerman[28] produced a deciduoma in 1 of 6 rats injected with testosterone as well as progestational changes in the endometrium. However, they attributed the decidual response to the numerous corpora lutea which were present in the ovary. Leathem and Crafts[29] produced progestational changes in the endometrium of spayed, adrenalectomized cats with desoxycorticosterone acetate, but did not attempt to produce deciduomas. Whether the source of the progesterone-like stimulus in case 1 was actually from the adrenal rest or from the adrenal cortex is conjectural. In neither case 10 nor case 16 was an adrenal rest found, and it is presumed that the decidual response was produced by progesterone elaborated in the adrenal cortex. It is also presumed that the occasional observation of decidual cells in the ovaries of postmenopausal women in whom no functioning corpus luteum remains can be explained on this basis. In none of these cases was adrenal or pituitary tissue available for study.

Summary

Sixteen examples of ectopic decidual reaction in the ovary in the absence of current or recent pregnancy are described.

In 14 of the 16 cases a functioning corpus luteum which had undergone disruption was present nearby; in one case the presence or absence of a corpus luteum could not be determined. An adrenal rest but no corpus luteum was observed in one case and in 2 cases nests of hilar cells were seen.

Periovarian adhesions were present in 14 cases; radiation therapy had been given in 9 cases; endometriosis was an associated condition in 5 cases.

The decidual reaction is defined as an inherent and unique property of the mesenchyme of the urogenital ridge and its derivatives. In the ovary the transformation usually occurs in an inconspicuous layer of cells normally located just below the mesothelial surface.

Decidual reactions in the absence of pregnancy are similar to experimentally produced deciduomas.

The decidual response follows stimulation of appropriate cells by

progesterone or progesterone-like substances elaborated usually in the corpus luteum, but in its absence, possibly in the adrenal cortex.

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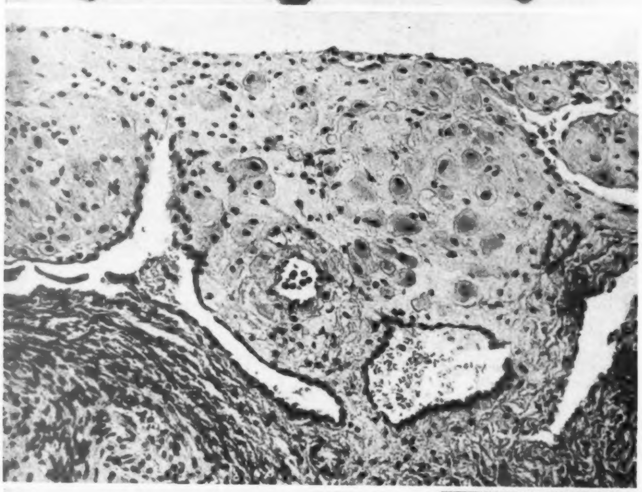
LEGENDS FOR FIGURES

FIG. 1. Case 13 (Armed Forces Institute of Pathology Acc. 582760). Elevated decidual nodules on ovarian surface. × 4½.

FIG. 2. Case 4 (A.F.I.P. Acc. 282199). Decidual cells in polypoid formation. Hematoxylin and eosin stain. × 150.

FIG. 3. Case 8 (A.F.I.P. Acc. 546894). Small nest of decidual cells adjacent to disintegrating corpus luteum. Hematoxylin and eosin stain. × 125.

1



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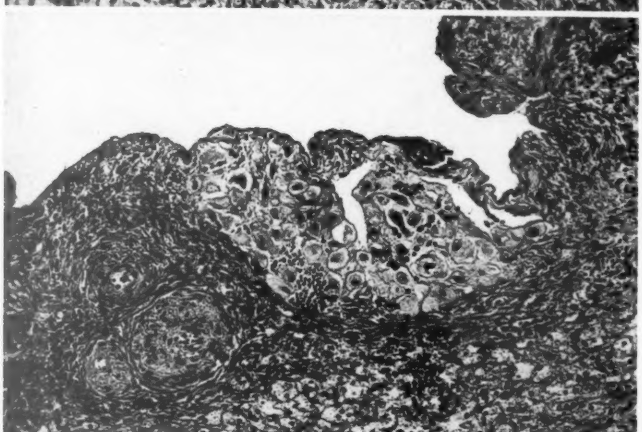

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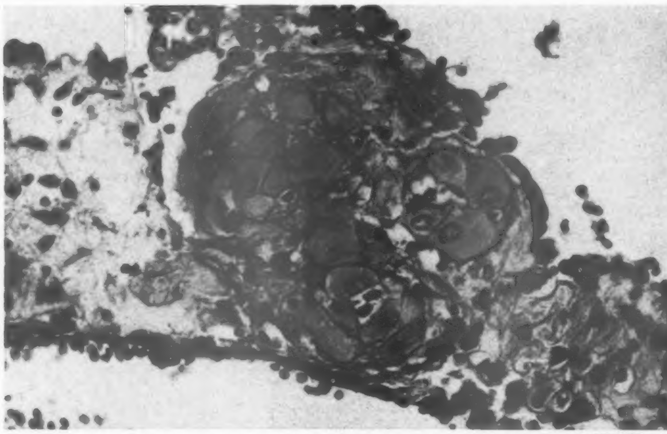

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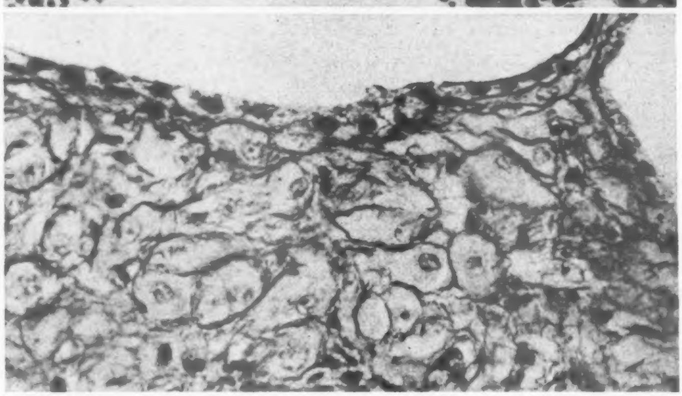


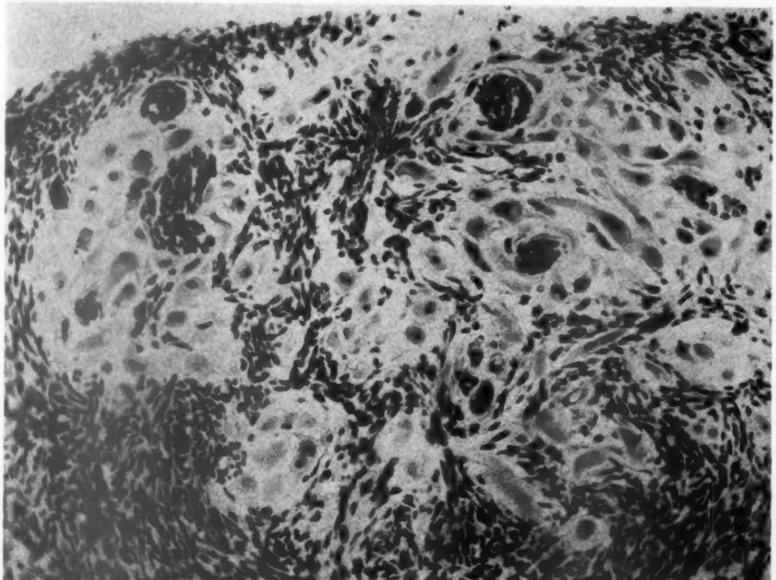
FIG. 4. Case 9 (A.F.I.P. Acc. 555513). Nest of decidual cells in inflammatory pannus. Periodic acid-Schiff (PAS) stain. × 275.

FIG. 5. Case 3 (A.F.I.P. Acc. 260099). Decidual cells are outlined by reticulum. Gridley's reticulum stain. × 300.

FIG. 6. Case 13 (A.F.I.P. Acc. 582760). Nest of decidual cells in cortical stroma. Hematoxylin and eosin stain. × 200.

FIG. 7. Case 10 (A.F.I.P. Acc. 564300). Scattered decidual cells in cortical stroma. Hematoxylin and eosin stain. × 300.

FIG. 8. Case 10 (A.F.I.P. Acc. 564300). The same group of cells as shown in Figure 7 with Schiff-positive outlines. PAS stain. × 350.



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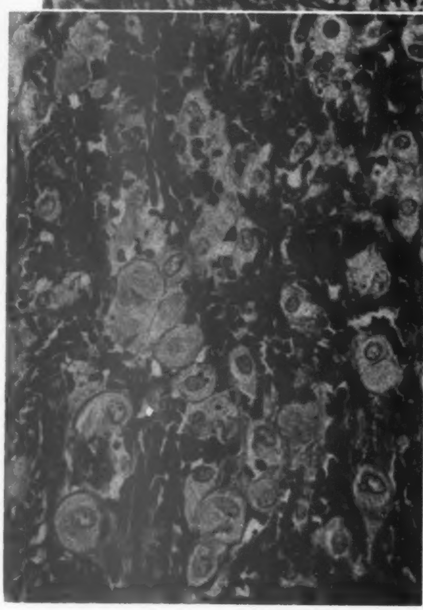

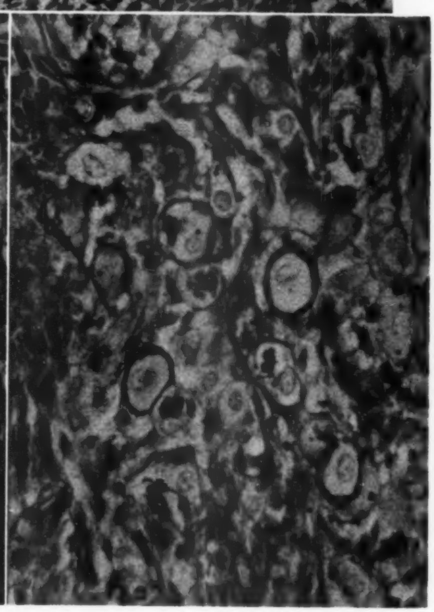

8

HISTOPATHOLOGY OF THE THYROID GLAND OF SHEEP IN PROLONGED ADMINISTRATION OF I^{131}*

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A study of the effects of the daily administration of I^{131} on sheep has been in progress at this installation for over 4 years.[1-3] Since I^{131} is an important product of atomic fission and is present in the gaseous wastes of chemical processes in the manufacture of plutonium, the investigation of its toxicologic effects when administered in small quantities for long periods is a matter of immediate interest. The primary purpose of this experiment was to furnish data on the maximum level of daily administration of I^{131} which is not productive of harmful effects in sheep, a range animal common to this geographic area. This data would also be of value in ascertaining similar levels in other large animals. During the course of this investigation an opportunity was afforded to observe pathologic effects resulting from such administration of I^{131} at various levels of dosage. The present report is concerned with the pathologic effects in the thyroid gland.

The pathology of the thyroid gland following the administration of I^{131} experimentally in single doses has been described in the dog,[4] rabbit,[5] rat, [6-8] and mouse.[9-11] The pathologic effects in thyroid tissue of man following the clinical use of I^{131} have been reported for patients with euthyroidism[12,13] and hyperthyroidism.[13-15] However, no studies involving the prolonged daily administration of I^{131} either clinically or experimentally have been reported.

METHODS

The principal phase of the experiment involved the daily oral administration of carrier-free I^{131} in feed pellets to Suffolk sheep. The method of administration insured ingestion of the total dose at each feeding. Details of the experimental methods have been described by Bustad *et al.*[16] in a separate report. Necropsies were performed on the animals after sacrifice by exsanguination under sodium pentobarbital anesthesia or after spontaneous death. Organs which

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showed obvious autolysis were deleted from the study. The tissues were fixed in Bouin's fluid and stained with hematoxylin and eosin. Mallory's aniline blue stain and Romeis' orcein stain were used when required.

In order to insure an adequate uptake of I^{131} by the thyroid gland and to simulate the dietary conditions prevailing in the environs, which are classified as marginally goitrogenic, the iodine content of the daily ration was limited. The total quantity of iodine in the daily ration was determined to be 0.3 to 0.5 mg., almost entirely in the form of stable I^{127}. The daily intake of I^{131} did not exceed 1.5×10^{-5} mg., which may be considered negligible.

To achieve the purpose of defining a maximum daily level of administration of I^{131} which produced no detectable abnormality, a wide range of feeding levels was originally employed, including doses of 0.005, 0.15, 5.0, and 240 μc. daily, as well as controls. Subsequent daily feeding schedules of 0.5, 1.5, 15, 30, and 45 μc. were added to narrow the range of uncertainty as to the maximum non-toxic level. Auxiliary experiments were conducted to explore problems of special interest, and these furnished additional pathologic data. The number of animals employed in the various feeding levels of this study appears in Table I.

TABLE I

Number of Animals and Amounts of I^{131} Administered Daily

	Number of control animals	Daily dose of I^{131} in μc.							
		0.15	5	15	30	45	135	240	480	1,800
Adults	16	12	9	3	6	0	0	10	16	3
Offspring	52	2*	18†	9	6	5	7	27	0	4

* Does not include 35 lambs sacrificed at or before 4 months of age.

† Does not include 39 lambs sacrificed at or before 4 months of age.

In an attempt to approximate conditions on the range for the purpose of evaluating the hazard to grazing sheep from prolonged contamination, the exposure was carried out throughout the cycle of reproduction. The original feeding of I^{131} to adult animals was continued through the period of gestation and lactation so that the offspring were exposed to I^{131} *in utero* and during the suckling period by way of the maternal milk. The daily oral feeding of I^{131} was then begun on the offspring at weaning (about 4 months of age) and continued for the duration of the experiment. This regimen provided the offspring with an exposure comparable to what they might be

expected to receive under natural conditions throughout their life cycle if their dams had been exposed, and, subsequently, they were exposed to the given quantity of I^{131}.

The inclusion of the fetal period and first year of life in the exposure of the offspring necessarily leads to a considerable variability in the intake of I^{131}. In the fetus this is dependent upon the quantitative partition of the ingested I^{131} between the ewe and fetus and the stage of functional development of the thyroid gland.[17] Subsequent to birth, it is dependent upon the quantity of milk ingested by the lamb and the concentration of I^{131} in the milk. After weaning, the intake is constant, since the lambs are then fed the spiked pellets. However, the ratio of I^{131} intake to body or thyroid weight is somewhat higher in the weaned lambs than in the adults.

The photomicrographs are accompanied by graphs of the variation of the concentration of I^{131} in the thyroid gland with time for each animal. The estimated cumulative thyroid gland exposures in rad units also are included. The following equation was used in the calculation of thyroid gland exposure:

$$\text{Dose (rad)} = \frac{51 \times E \times q \times t}{W}$$

$51 =$ disintegrations/μc./day $\times$ ergs/mev $\times$ rad/erg
$E =$ average energy of beta particles in mev
$q =$ mean of quantity deposited in thyroid gland in μc. as determined by external monitoring or estimated in the case of *in utero* exposure
$t =$ days exposed to I^{131}
$W =$ weight of thyroid gland in grams estimated on the basis of average organ weights at necropsy for various ages and seasons

This formula assumes that all of the energy of the beta particles is absorbed within the thyroid gland, ignoring the loss to surrounding tissue. The gamma component was ignored since it was estimated to be less than 10 per cent of the beta component. In view of the number of inexact estimates required, the calculated dosages are considered to be crude approximations. Periods of feeding during which measurable amounts of radioiodine were not concentrated because of thyroid ablation were deleted from the graphs.

Results

The variation of I^{131} intake of the offspring compared with the adult as well as differences in response between mature and immature tissues make it necessary to describe the findings in the adult animals separately from their progeny in several instances.

Histology of the Normal Thyroid Gland of Sheep

The normal thyroid gland of the sheep shows the basic pattern observed in the human and in common experimental animals. The follicles are intimately apposed to each other, being separated by a rich capillary network, which is not readily apparent in the microscopic sections, and a delicate reticular stroma. Lability of follicular epithelium is demonstrated by the variation in histologic patterns, which range from that of large follicles with low cuboidal epithelium to small follicles lined by high columnar epithelium and with peripherally vacuolated colloid. The nuclei are usually round or oval and contain finely dispersed chromatin. Fibrous septa of moderate size, which contain blood vessels and nerves, traverse the parenchyma. The periphery of some of the adult thyroid glands shows fibrous tissue between the follicles, a finding which is most striking in the outermost layers and present only in relation to septa in central portions of the glands. This normal histologic feature was a source of difficulty in evaluating slight damage, since fibrosis was the principal evidence of damage in the adults of the groups receiving low levels of I^{131}.

A high percentage of the normal glands contains islands of stratified squamous epithelium which are frequently cystic, resembling small epidermoid cysts of human skin (Fig. 1). Parathyroid tissue frequently is included and thymic tissue rarely so (Fig. 2). Lymphocytic infiltrations and adenomatous nodules are not encountered in the normal gland.

Adult Sheep Administered 0.15 to 5.0 μc. of I^{131} Daily

Damage was not observed histologically in animals receiving 0.15 μc. of I^{131} for a maximum period of 4 years. Members of the groups introduced later at levels between 0.15 and 5.0 μc. as well as offspring of the group fed 0.15 μc. of I^{131}, of an age greater than 6 months, have not yet been sacrificed.

The lowest daily dose productive of damage was 5.0 μc.[18] Seven of 9 adult animals in this group showed interfollicular fibrosis, which was the principal pathologic effect observed. The fibrosis was slight in six glands and mild in one (Fig. 3). In view of the previously mentioned normal occurrence of fibrous connective tissue between follicles in the periphery of the glands and adjacent to septa in deeper locations, especially in older animals, differentiation between the fibrous connective tissue observed as a feature of the histology of

the gland and that occurring pathologically was extremely difficult in cases of minimal damage. As a manifestation of damage, the fibrous tissue had a random distribution and a compact texture. When present in minimal form, the distribution of the scar tissue was focal, but the generalized nature of the distribution became obvious with the developing severity of the process. The only deviation from the normal pattern in the epithelium was the presence of a rare enlarged nucleus. However, there was a diminution in the average size of the follicles in affected glands. The presence of damage could not be established in two glands.

Vascular changes of a minimal character were observed in a few glands of this group. The damaged arteries were of medium size and showed focal thickening of the intima due to fibrosis, with splitting and fraying of the internal elastic lamina. The media and adventitia were unchanged, and the arterioles showed no pathologic alteration.

The initial damage was observed at 35 months of feeding. Animals of this group which expired prior to this age exhibited significant autolysis and were not included in the results. For this reason 35 months may not represent the minimal period of feeding of 5.0 μc. of I^{131} per day at which morphologic effects can be initially observed in an adult animal.

Offspring Administered 5.0 μc. of I^{131} Daily

Tissue damage was observed in the thyroid glands of 12 of 15 offspring, 8 months of age or older, of animals which received 5.0 μc. of I^{131} daily. The principal effect was focal interfollicular edema in contrast to the fibrosis described in the adults. Within edematous areas the follicles were rounded and appeared to be suspended in a fluid medium (Fig. 4). In early cases, the interfollicular fluid was essentially devoid of fibers, but a fibrous component appeared in the glands exposed for longer periods. A few infiltrations of lymphocytes were observed in some glands.

Intermediate Feeding Groups

The thyroid glands of 3 ewes fed 15 μc. of I^{131} per day for a period of 2 years showed generalized minimal changes. Fibrosis was present between the follicles and was loose and edematous in one gland. The pattern of the follicles was undisturbed. However, the nuclei of the epithelial cells showed some variation in size, with slight clumping of the chromatin to form small chromatin knots. The arterial effects, which were questionable in the ewes fed 5.0 μc. per day, were more

definite in animals fed 15 μc. The intima of certain arteries showed distinct fibrous thickening.

Two offspring of the ewes fed 15 μc. per day, sacrificed at 4 months of age, had normal thyroid glands. Four lambs which were sacrificed at 15 months of age showed edema and fibrosis of moderate severity. Occasional lymphocytes were observed in the stroma. Two of three thyroid glands of lambs born to the same ewes a year later and sacrificed at 4 months showed damage, primarily edema.

Six ewes at 20 months of age received 30 μc. per day of I^{131} for a period of 6 to 7 months. All members of this group showed minimal evidence of damage. Slight interfollicular fibrosis was observed in five glands. The sixth gland revealed a small accumulation of lymphocytes without evident fibrosis.

Six offspring of ewes fed 30 μc. per day of I^{131} were exposed *in utero* during the last two trimesters of pregnancy and subsequently during the period of suckling. They were sacrificed at periods between 10 and 130 days after birth. The thyroid gland of the lamb sacrificed 10 days after birth was normal. The thyroid glands of the remaining 5 lambs, including one sacrificed at 29 days, were damaged, all but one showing edema. Fibrosis and lymphocytic infiltration were associated findings in two glands. Only fibrosis was encountered in the fifth gland.

Five offspring of ewes fed 45 μc. of I^{131} per day showed damage to the thyroid gland. Two lambs were newborn, and the remaining 3 were sacrificed at 4 months of age. Of the 2 newborn lambs, one showed minimal and the other severe edema. Edema also predominated in the glands of the 4-month-old lambs, being accompanied by mild fibrosis in one and by slight inflammation in another.

Seven offspring of ewes fed 135 μc. per day showed thyroid damage. Three lambs were newborn, 2 were 1 month, and 2 were 4 months of age. Edema of mild to moderate severity was present in all glands, being accompanied by fibrosis in 2 of the 7 lambs.

Adult Sheep Administered 240 μc. of I^{131} Daily

The effects of prolonged administration of high levels of I^{131} were observed in the group of 2-year-old animals which received 240 μc. per day for periods up to 450 days.[10] Two animals of the group died during the period of administration while 8 others died or were sacrificed subsequent to the termination of I^{131} feeding.

The first necropsy on an animal of this group was performed 6 months after the feeding was initiated. Microscopically, the gland

showed a widespread separation of follicles by fibrous tissue. The fibrous tissue was predominantly mature and occasionally had an acellular, hyalinized appearance, although loose, edematous areas were noted also. Inflammation in the fibrous stroma consisted of scattered infiltrations of lymphocytes. The follicles showed a moderate reduction in size in central areas. The epithelial cells generally were normal in appearance, apart from a mild variation in nuclear size with slight condensation of chromatin. A few cells showed hydropic degeneration of the cytoplasm, causing a ballooned appearance. Similar desquamated cells were present in the lumina of a few follicles.

After a year of feeding, the thyroid gland of a second ewe showed dense fibrosis. The follicles were substantially reduced in size and showed a frequent absence of distinct lumina. The epithelial cells varied in size and shape. Nuclear irregularity was prevalent, many nuclei being moderately enlarged with prominent chromatin knots. A central pallor was observed in some nuclei while others were pyknotic. The fibrotic stroma contained scattered neutrophilic leukocytes, their presence at this advanced stage of damage being an isolated finding in this gland.

The administration of I^{131} in a daily dose of 240 μc. was continued for 450 days (about 15 months). No essential difference was observed in the histologic pattern between those animals which died or were sacrificed at the end of 15 months and others which survived after the cessation of feeding of I^{131} for periods up to 24 months. A marked reduction in the quantity of surviving parenchyma with the residual follicles surrounded by hyalinized fibrous tissue was the usual finding in this group of severely damaged thyroid glands. The follicles were markedly constricted, although a few follicles at the surfaces of the glands were of normal size and contained colloid. Large central areas were completely devoid of epithelial cells, the parenchyma being entirely replaced by the fibrous connective tissue. The epithelial cells showed a more advanced degree of alteration than that observed at earlier intervals. Many nuclei were enlarged and displayed a coarse chromatin structure. However, no bizarre or gigantic cells were observed. Inflammation was virtually absent. One gland displayed small areas of necrosis which had an amorphous, glazed appearance. One such focus was surrounded by a layer of foreign body giant cells. These distinctive necrotic areas may have been derived from epidermoid cysts.

Vascular effects were pronounced in the thyroid glands of this

group. The intimal fibrosis and changes in the internal elastic membrane mentioned previously were accentuated. The intimal thickening was present in various stages of severity, culminating in complete obliteration of the lumen. In the earlier stages, the thickened intima was moderately cellular, some of the cells being pyknotic or distorted. Subsequently, the nuclei disappeared, leaving only the connective tissue fibers. The internal elastic lamina had a frayed or disorganized appearance. Later, a gradual attenuation of the smooth muscle in the media occurred. The adventitia was fused with the surrounding hyalinized fibrous tissue. In the end stage of damage the vessels could be distinguished only by remnants of elastic tissue which formed crude outlines of the pre-existing arteries.

Evidence for the importance of the direct action of radiation on arteries was obtained in 2 specimens of this group. In both cases large arteries immediately external to the thyroid glands of ewes fed 240 μc. of I^{131} for prolonged periods showed asymmetric damage. In the cross sections of each artery, the proximal half showed typical severe damage while the distal half showed a gradation to a normal appearance. One artery showed severe endothelial proliferation in the intima and patchy areas of loss of nuclear stain in the media of the proximal half (Fig. 5). In the second artery there was a loss of nuclear detail throughout the thickness of the media of the proximal segment. The adventitia was not obviously altered in either vessel. The restriction of the pathologic effects to the proximal regions in the vessels is attributed to the limited range of penetration of the beta particles.

Offspring Administered 240 μc. of I^{131} Daily

The dosimetric considerations in the offspring of ewes fed 240 μc. of I^{131} per day were complicated by the fact that the administration was begun at the onset of the third trimester of pregnancy. The first-year offspring thus received I^{131} via the placenta only during the last third of fetal life; after birth they were exposed further by way of the maternal milk and, after weaning, by direct feeding. None of the first-year offspring survived beyond 17 months of age. The original ewes were already severely hypothyroid at the time of delivery of the second-year offspring, and the lambs were exposed to I^{131} throughout their fetal life. This group of lambs was either stillborn or lived a maximum period of 5 days.

The changes observed in the first-year offspring were similar generally to those described in the adult. However, more specimens

illustrating the earlier stages of damage were available in the offspring than in their dams. Two animals which died at birth showed no abnormal findings in the thyroid gland. Three lambs were sacrificed at 4 months of age. One gland showed central areas of necrosis and replacement fibrosis. Surrounding these areas a relatively abrupt transition to thyroid follicles having a normal configuration occurred, the surviving parenchyma comprising the bulk of the cross section of the gland (Fig. 6). The follicles were closely arranged in the periphery and revealed cellular abnormalities. The cells were distorted, and the nuclei showed clumping of chromatin. The second gland showed fibrosis between follicles of normal size in the periphery with necrosis of the center of the gland. Small lymphocytic infiltrations were observed. Necrosis was absent in the third gland, but loose fibrosis between follicles was uniform throughout the sections. The follicles were only slightly reduced in size in central areas. Lymphocytes were scattered widely throughout the stroma and were accompanied by a few neutrophilic leukocytes. A mode of destruction of follicles by localized rupture of the follicular wall (Fig. 7) and foreign body giant cell reaction to follicular disintegration (Fig. 8) were illustrated in this thyroid gland. The three glands showed a variation of effects ranging from complete central necrosis or fibrosis with no peripheral fibrosis to an absence of necrosis with diffuse interfollicular fibrosis.

The thyroid glands of 7 lambs which subsequently died spontaneously at various ages up to 17 months showed more severe damage. Fibrosis was dense, and the follicles were markedly constricted. The surviving microfollicles often had a minute lumen or none at all (Fig. 9). The cells showed no evident cell boundaries, the cytoplasm merging into the enveloping fibrous tissue. The nuclei usually were somewhat larger than normal and showed clumping of chromatin. Vascular changes were similar to those described in the thyroid glands of the adult animals fed 240 μc. per day (Fig. 10).

Twelve second-year offspring of the original ewes fed 240 μc. per day, which were exposed to I^{131} via the fetal circulation throughout their fetal life, were stillborn or died during the neonatal period. The thyroid glands were reduced in size. The majority of the follicles were inordinately small in comparison with those of a normal neonatal gland, the lumina being minute. However, follicles of normal size often were present in the periphery of the glands. Interfollicular edema was observed in central areas (Fig. 11) and was not accompanied by fibrosis or inflammation.

Adult Sheep Administered 1,800 μc. of I^{131} Daily and Their Offspring

Identical histologic patterns were observed in the thyroid remnants of adult ewes fed 1,800 μc. of I^{131} daily for 5 and 14 months. The gland in each case was replaced by a hyalinized fibrous scar with only a few scattered, shrunken, and distorted follicles remaining in the periphery. A third ewe received the same amount daily for 14 months but was not sacrificed until 19 months after the cessation of I^{131} feeding. The gland of this animal showed a marked fibrous obliteration. The epithelial remnants consisted of a few solitary cells and minute follicles within the hyalinized scar (Fig. 12).

Thyroid glands were obtained from 4 offspring of the ewes fed 1,800 μc. per day, 2 being stillborn, one having lived for 15 hours, and one for 3 days. The pathologic effects were severe despite the fact that the fetal exposure to I^{131} occurred only during the last trimester of pregnancy. Necrosis was observed in central areas, being accompanied by deposits of blood pigment (Fig. 13). Fibrosis was observed in both central and peripheral locations.

Special Studies on Adult Sheep Administered 480 μc. of I^{131} Daily

Two secondary experiments were performed utilizing a daily dose of 480 μc. of I^{131}. The purposes of the first experiment were the determination of the shortest period of administration required to cause histologic evidence of damage and the observation of the early histopathologic effects at this level.[20] In order to achieve this objective, 7 yearling rams were used as experimental subjects and were sacrificed after 15, 19, 25, 29, 36, 43, and 70 days of feeding, respectively.

The thyroid gland of the sheep sacrificed at 15 days was normal. The first evidence of damage was observed at 19 days. Focal inflammation was present within the parenchyma, the inflammatory cells being lymphocytes and a few neutrophilic leukocytes. Small groups of cells suggestive of epithelial fragments were observed occasionally in the midst of the inflammation. The margins of these areas showed an invasion of inflammatory cells, principally neutrophils, into the stroma between the neighboring follicles, with an early encroachment upon the follicles. The adjacent follicles occasionally contained desquamated epithelial cells within the lumina.

At 25 and 29 days, similar infrequent foci of inflammation were observed (Fig. 14). However, the severity of the inflammation had

increased substantially at 36 days, the areas of involvement being numerous and large. Slight fibrosis was present between follicles in areas devoid of inflammation. Early vascular damage was apparent.

At 43 days, the damage was diffuse, the follicles throughout the central areas being separated by a loose, edematous stroma containing scattered neutrophilic leukocytes and fibroblasts. An additional feature was the presence of multiple small infarcts. The severity of the process was less pronounced in the animal sacrificed at 70 days, presumably because of an individual diminished uptake of I^{131}.

The second experiment conducted at this level of administration of I^{131} was performed in order to observe differences in the effects encountered after withdrawal of the I^{131} in contrast to those observed after its continued administration. A second objective of this experiment of a clinical character was a comparison of changes in the ratio of serum iodide to protein-bound iodine (conversion ratio), in the external counting of the activity in the thyroid gland, and in the histology of the thyroid gland in early damage.[21] A group of 9 yearling rams was divided into three subgroups of 3 animals each. The first subgroup was fed I^{131} until clinical evidence of thyroid damage was obtained either by a decline in the iodide conversion ratio or in the external thyroid count, and then sacrificed. The members of the second subgroup were sacrificed after being maintained on the feeding for a period of 60 days after damage was considered present by one of the two methods. The third subgroup was taken off I^{131} feeding when the conversion ratio or thyroid count declined, but was not sacrificed until 60 days later. A progressively diminishing radiation exposure occurred in the third group, permitting a slight opportunity for recovery or stabilization of tissue damage.

The members of the first subgroup were sacrificed at periods of 43, 44, and 57 days, respectively, after the beginning of the experiment. The three glands showed a generalized separation of follicles of normal size by loose fibrous connective tissue. Lymphocytes and neutrophilic leukocytes were scattered in the stroma. Desquamated epithelial cells often were present in the lumina of the follicles. One gland also revealed multiple small infarcts (Fig. 15).

In the second subgroup, the subjects were sacrificed at the end of 86, 104, and 108 days, respectively, having received I^{131} continuously throughout these periods. One gland showed fibrosis with fairly good preservation of the follicles apart from large, sharply delimited infarcts. In the second gland, degeneration and distortion of the follicles were apparent in addition to areas of fibrosis and infarcts. Early

calcification was evident within infarcts. The third gland revealed widespread necrosis, the majority of the follicles being destroyed although their outlines were evident. Within the large areas of necrosis, collections of preserved giant cells were observed (Fig. 16). The giant cells were variable in size, each containing several to many nuclei in a homogeneous, eosinophilic mass of cytoplasm with irregular margins. Detritus was present within niches in the boundaries of giant cells.

The members of the third subgroup were sacrificed 60 days after a decline in the external thyroid count or conversion ratio provided objective evidence of thyroid damage, as has been described. No I^{131} was administered during the 60-day period, and the animals were sacrificed 94, 115, and 121 days after the onset of the experiment. The damage was only slightly less than that observed in the second subgroup. One gland showed generalized, moderately severe fibrosis without necrosis. Scattered foci of necrosis were present in the midst of fibrotic but reasonably well preserved thyroid tissue in the second gland. The third gland showed extensive necrosis with the presence of giant cells, the degree of damage being comparable to that of the most severely affected gland in the second subgroup.

Discussion

The various elements of pathologic damage observed in this experiment are similar to those previously described in the clinical and experimental literature. However, the variety of controlled conditions employed permits a degree of correlation of specific effects with such factors as age and levels of dosage. Secondly, the general histologic patterns differ in certain respects in single dose and prolonged daily administration of I^{131}.

The interstitial tissue was a more obvious site of pathologic change in the thyroid gland in this experiment. Although the epithelial effects are probably responsible for the functional decline of the gland, the diagnosis of early thyroid damage due to prolonged exposure to I^{131} at low levels would best be attempted on the basis of the manifestations in the interstitial tissue.

The most characteristic tissue response in the thyroid gland of the young animal was edema. It was encountered at birth in fetuses exposed to high daily levels of I^{131} *in utero,* during the nursing or yearling periods in animals fed intermediate levels of I^{131}, and was the most frequent finding as late as the second year of life in the offspring of the group fed 5.0 μc. of I^{131} daily.

The microscopic appearance in edematous areas occasionally resembles that of the human fetal adenoma. A question exists as to whether the stroma in the fetal adenoma represents a true edema or a fluid matrix of different composition. The same question may be applied to the edematous appearing stroma in the pathologic material of this experiment. Since means of investigating the chemical composition of the fluid were not available, no statement can be made as to its true chemical nature. In spite of the histologic resemblance, an identity with the matrix of the fetal adenoma cannot be immediately assumed in view of the dissimilarity in the circumstances of development of the stroma. The stroma of the fetal adenoma is a feature, possibly degenerative, of a benign tumor or proliferative nodule whereas the edematous stroma encountered in this experiment is an element of a response to a traumatic agent, ionizing radiation. The stroma in the fetal adenoma is variously called edematous, a matrix, or an unknown substance; the term edema has been used in this report to characterize the fluid stroma encountered in our material. However, the reservation must be maintained that this stroma is of an unknown composition, which may differ from that of the broad and poorly defined category, edema fluid.

Interstitial fibrosis was the prominent feature of the pattern of damage in the adult animal. It may be assumed that the fibrosis represents the end stage of preceding inflammatory or necrotizing processes. However, at the lowest injurious level, the group fed 5.0 μc. of I^{131} daily, the earlier stages postulated were not detectable, so that the fibrosis in effect assumed the significance of an initial indicator of damage. At higher levels of dosage, it was a more obvious sequel to inflammation or necrosis.

At the high level of daily administration of 480 μc. of I^{131}, foci of inflammation in the interstitial tissue constituted the earliest observed effect. The early development of inflammation may also be expected at other high or intermediate levels at which inflammation frequently was observed in association with edema or fibrosis.

The manifestations of epithelial damage ranged from slight nuclear abnormalities to extensive necrosis. Minimal nuclear alterations, such as clumping of chromatin and changes in nuclear size, developed concomitantly with the early interstitial effects. However, the progression of the epithelial changes was relatively slow, making it difficult to evaluate the importance of primary as opposed to secondary epithelial damage. The former would stem from the direct action of

ionizing radiation, and the latter from mechanical constriction by scar tissue and from impairment of blood supply due to vascular damage. The foci of inflammation observed early in high-level feeding may constitute a response to primary epithelial damage, and early central necrosis would certainly seem to be a direct radiation effect. Evidence for a constrictive mechanism is furnished by a correlation between the diminution in follicular size and the development of scar tissue. The occurrence of infarcts dramatically demonstrates one effect of vascular impairment. A phenomenon of potentially greater physiologic significance at low levels is the deposition of interfollicular fibrous tissue, interposing a possible barrier to exchange between the follicles and their capillaries. It seems likely that both primary and secondary damage contribute to the loss of functional epithelial tissue.

Pronounced cellular changes were observed in this experiment, especially in the presence of severe damage, but truly bizarre cells were not evident. Goldberg and Chaikoff[22] described the occurrence of carcinoma of the thyroid gland in rats 18 months after single large doses of I^{131}. No tumors were encountered in the thyroid glands of sheep in this experiment after a maximum period of 4 years.*

Although 5.0 μc. of I^{131} per day represents the lowest level causing damage in the necropsy material accumulated thus far, it is unlikely that 5.0 μc. represents the absolute minimal toxic dose. An absolute minimal toxic level would be one causing a demonstrable effect only at the end of the animal's life span, the effective life span of the sheep being 6 to 7 years under experimental conditions. If any feeding level causes damage at a substantially earlier age, one may assume that a lower dose exists which would cause initial manifest damage at a later period in the animal's life. On the basis of the existing results, one may conclude that 0.15 μc. of I^{131} per day administered initially to the adult sheep is not injurious. However, the offspring of the group receiving 0.15 μc. and members of the groups fed 0.5 and 1.5 μc. have not yet reached the age chosen for sacrifice. In view of the observation of pathologic effects in the offspring of ewes fed 5.0 μc. at an age as early as 8 months, the occurrence of damage at a later age in the offspring of a group fed less than 5.0 μc. may be expected.

*In the group fed 5.0 μc. of I^{131} daily, a metastasizing fibrosarcoma has developed in or immediately adjacent to the thyroid gland of a 52-months-old ewe offspring during the fifth year of the experiment and will be reported separately.

Summary

The pathologic effects in the thyroid glands of sheep receiving prolonged daily oral administration of I^{131} are reported. Levels of dosage employed ranged from 0.15 μc. to 1,800 μc. per day.

No pathologic alterations were observed with a daily feeding level of 0.15 μc. after a maximum period of observation of 4 years. The principal early manifestations of thyroid damage were interfollicular fibrosis in adults receiving 5.0 μc. daily and interfollicular edema in their offspring. Focal inflammation was the initial effect resulting from a daily dose of 480 μc. At feeding levels between 5.0 and 480 μc. per day, various combinations of edema, inflammation, and fibrosis occurred. Prolonged administration of higher levels of I^{131} caused necrosis of follicles or infarcts. Progressive arterial damage was observed, including intimal thickening, changes in the internal elastic membrane, destruction of the smooth muscle of the media, and eventual obliteration of the lumina.

The pathologic changes observed involve the interstitial tissue, epithelium, and blood vessels. The effects on the interstitial tissue may be considered of primary significance in the early diagnosis of prolonged exposure to low levels of I^{131} in the species investigated.

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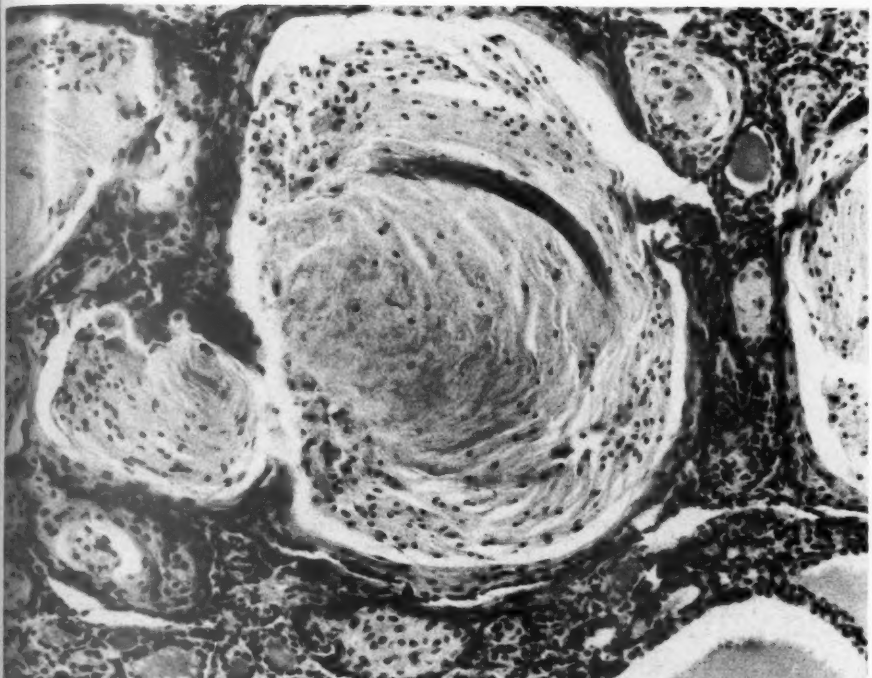
LEGENDS FOR FIGURES

FIG. 1. Cystic islands of stratified squamous epithelium in a normal thyroid gland of sheep. Hematoxylin and eosin stain. × 125.

FIG. 2. Internal parathyroid gland and ectopic thymic tissue within normal thyroid gland. Parathyroid tissue partially surrounds thymic tissue. Hassall's body is present within thymic tissue near the upper margin. Hematoxylin and eosin stain. × 125.



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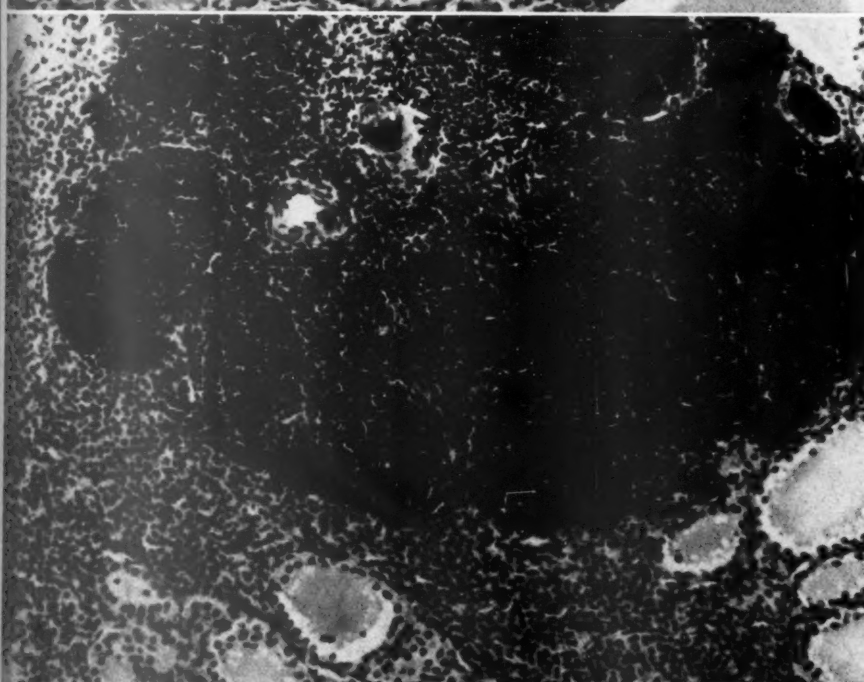

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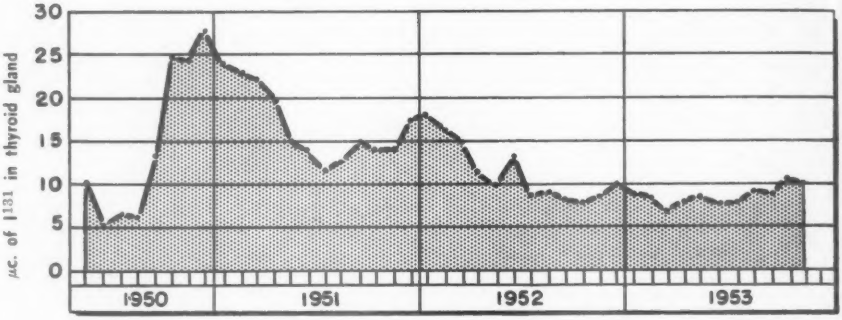
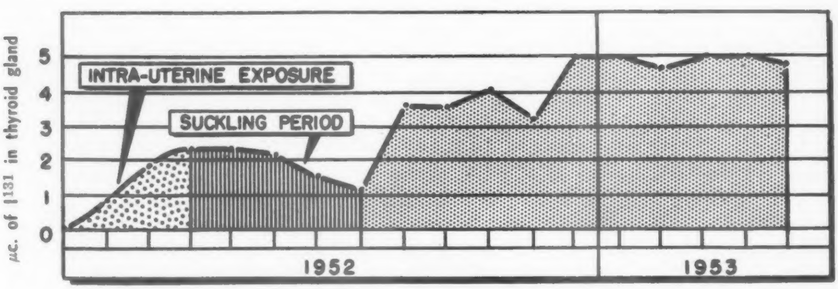


Period of exposure (months)
Chart 1



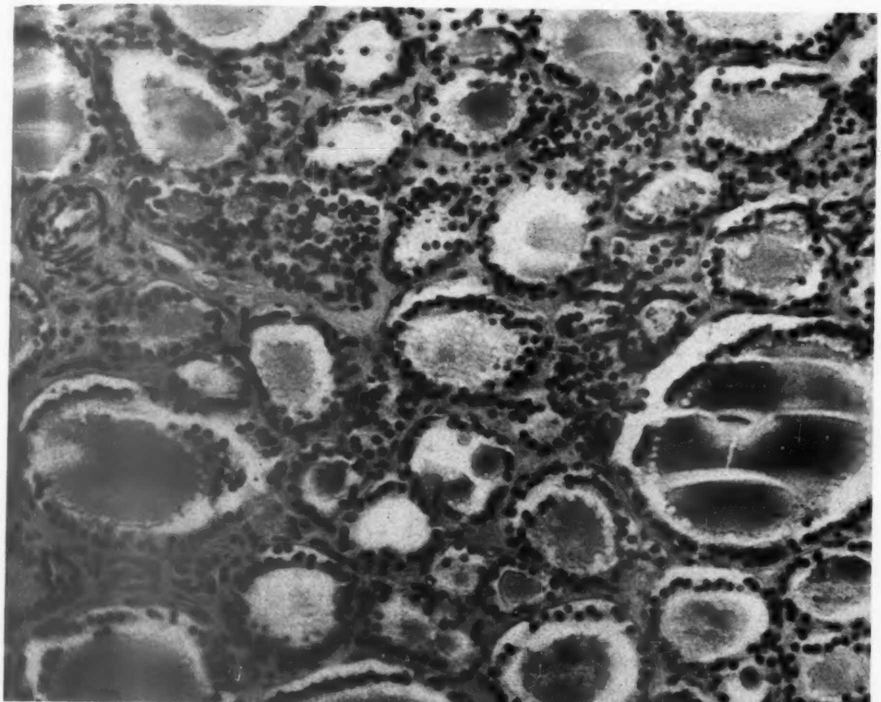
Period of exposure (months)
Chart 2

CHART 1. Quantity of I^{131} in thyroid gland depicted in Figure 3 (estimated total radiation dose, 26,000 rad).

FIG. 3. Mild interfollicular fibrosis in a thyroid gland of an adult ewe receiving 5.0 μc. of I^{131} per day for 43 months. Hematoxylin and eosin stain. × 150.

CHART 2. Quantity of I^{131} in thyroid gland depicted in Figure 4 (estimated total radiation dose, 10,000 rad).

FIG. 4. Moderately severe edema between follicles in the thyroid gland of a 14-months-old offspring of a sheep fed 5.0 μc. of I^{131} daily. Hematoxylin and eosin stain. × 275.



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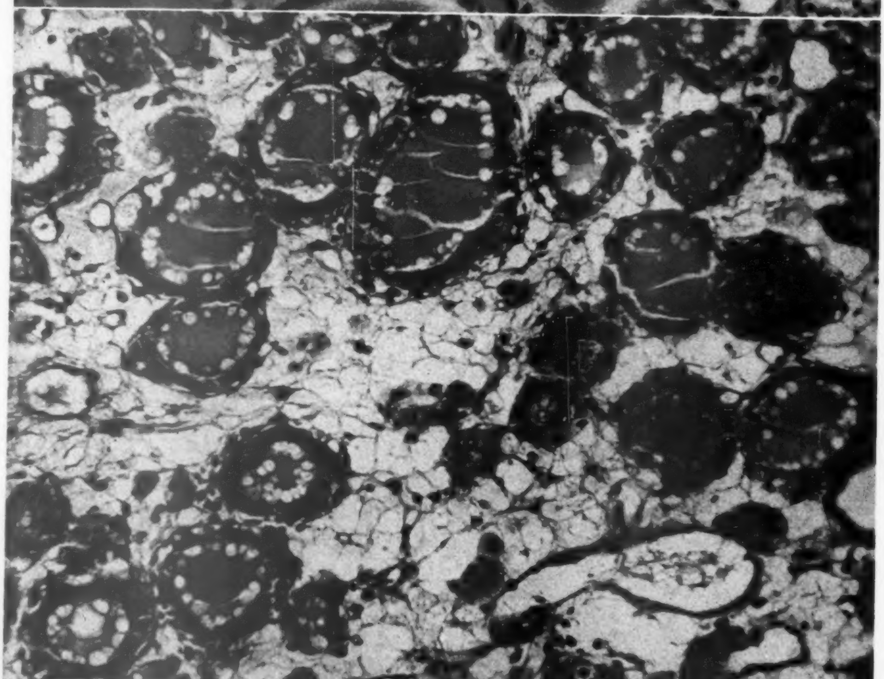

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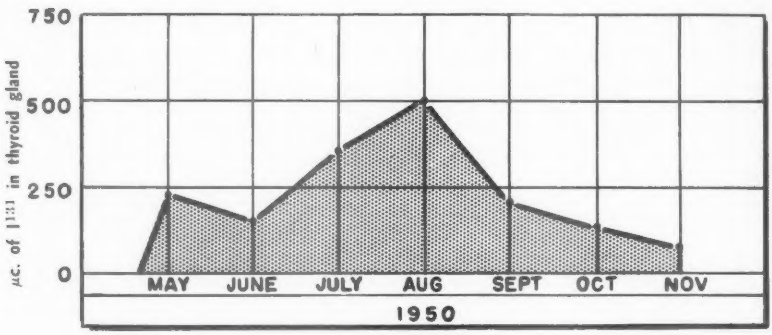
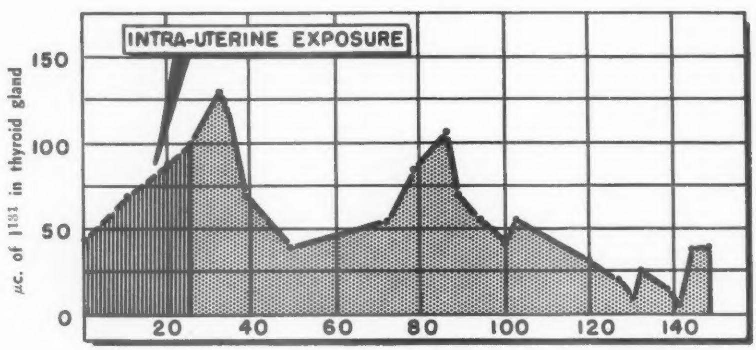


Period of exposure (months)
Chart 3



Period of exposure (days)
Chart 4

CHART 3. Quantity of I^{131} in thyroid gland depicted in Figure 5 (estimated total radiation dose, >70,000 rad).

FIG. 5. Large artery external to the thyroid gland showing severe intimal thickening and focal fibrosis of media in the proximal half of the vessel (lower left). From an adult ewe which received 240 μc. of I^{131} per day for 450 days. Hematoxylin and eosin stain. × 25.

CHART 4. Quantity of I^{131} in thyroid gland depicted in Figure 6 (estimated total radiation dose, 60,000 rad).

FIG. 6. Central necrosis and fibrosis with less damage in the periphery of the thyroid gland of a 120-day-old offspring of a ewe fed 240 μc. of I^{131} per day. Hematoxylin and eosin stain. × 70.

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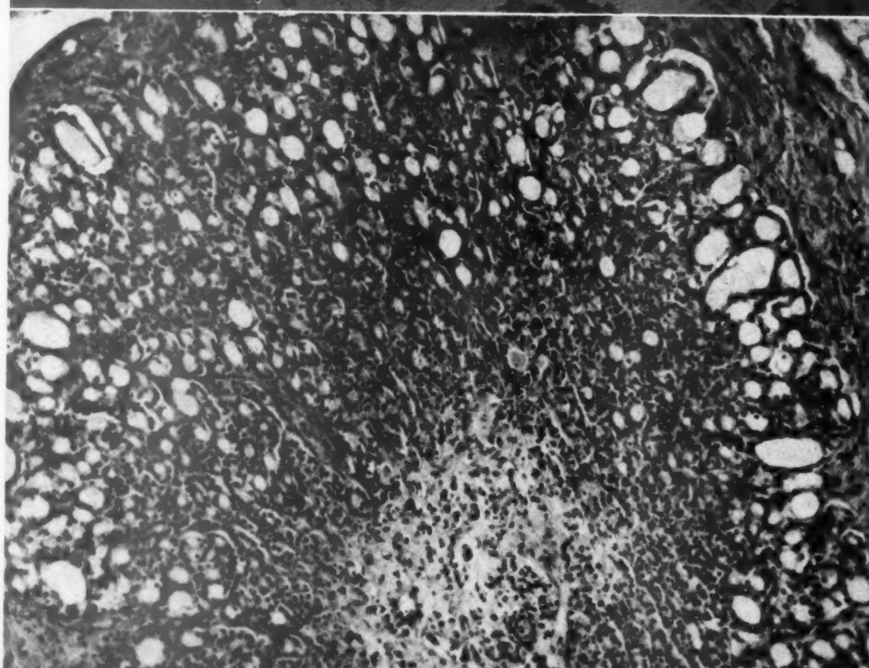

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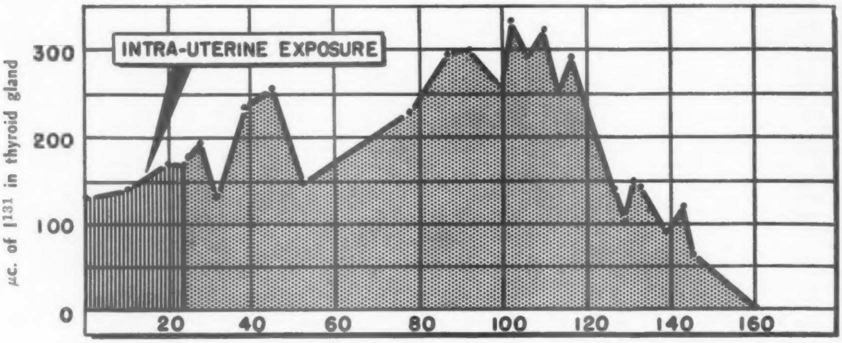
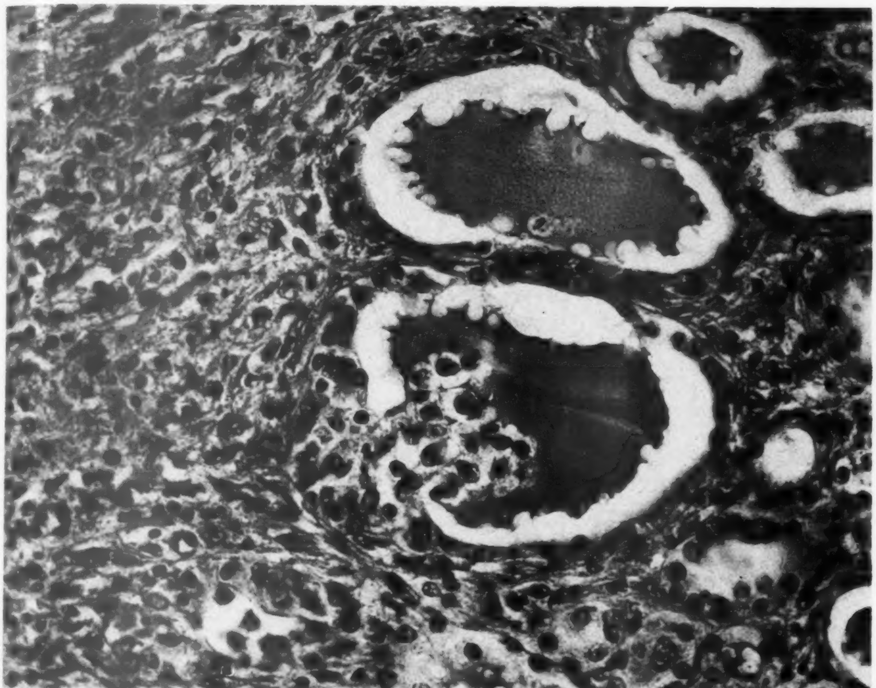


Chart 5

CHART 5. Quantity of I^{131} in thyroid gland depicted in Figure 7 (estimated total radiation dose, 100,000 rad).

FIG. 7. Minimal fibrosis and chronic inflammation in the thyroid gland of a 7-months-old offspring of a ewe fed 240 μc. of I^{131} daily. This is an early stage in the disintegration of a follicle by rupture of the follicular wall and invasion of epithelial and stromal cells into the lumen. Hematoxylin and eosin stain. × 300.

FIG. 8. Giant cells within the focus of inflammation at the site of disintegration of a follicle in the thyroid gland shown in Figure 7. Hematoxylin and eosin stain. × 300.



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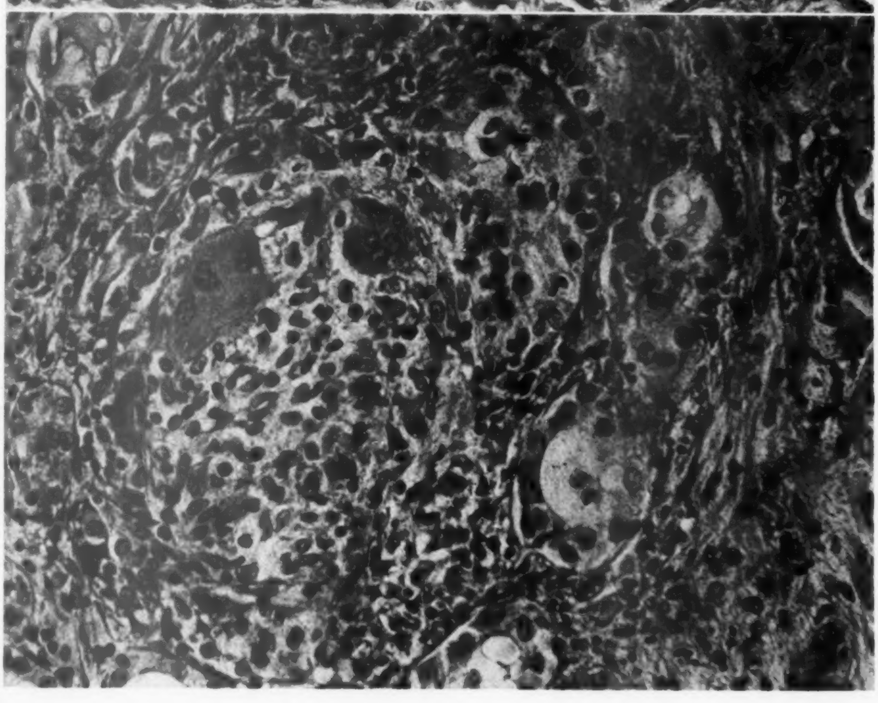

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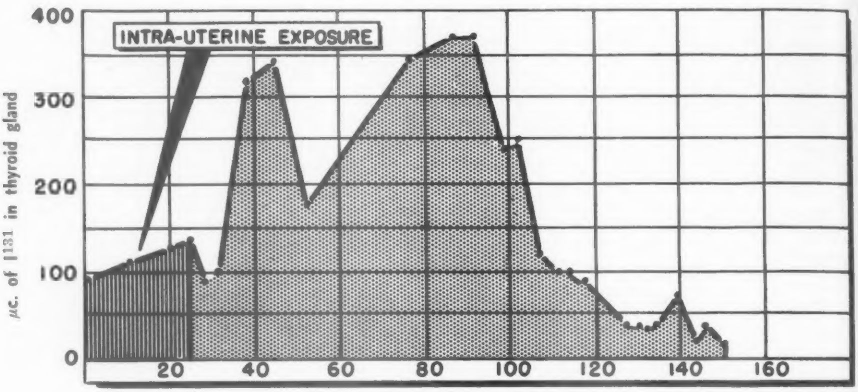


Chart 6

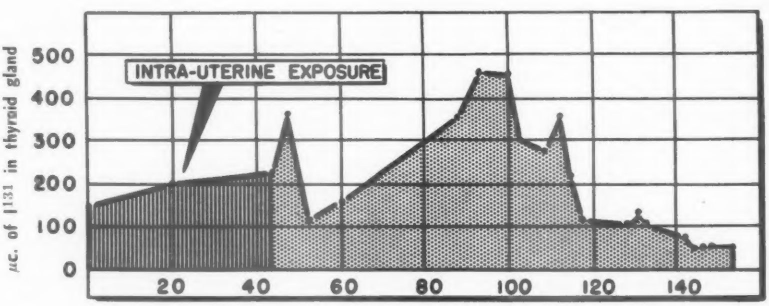


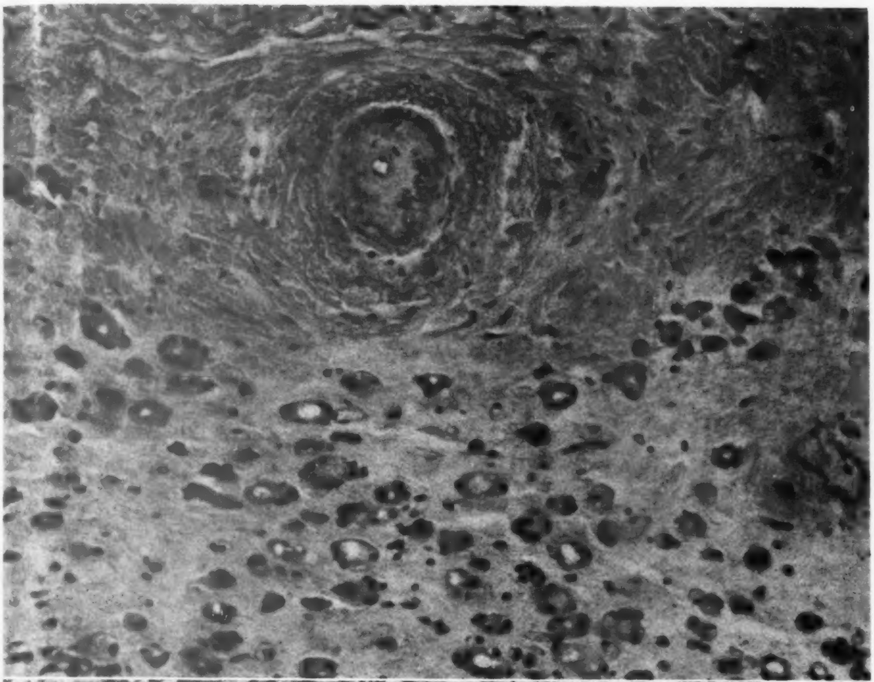
Chart 7

CHART 6. Quantity of I^{131} in thyroid gland depicted in Figure 9 (estimated total radiation dose, >100,000 rad).

FIG. 9. Microfollicular pattern within dense fibrous tissue in an 11-months-old offspring of a ewe fed 240 μc. of I^{131} daily. Arteries are severely damaged. Only the elastic tissue remnants of a vessel near the right margin of the photomicrograph are visible. Hematoxylin and eosin stain. × 150.

CHART 7. Quantity of I^{131} in thyroid gland depicted in Figure 10 (estimated total radiation dose, >100,000 rad).

FIG. 10. Intimal thickening with narrowing of the lumen of a small artery in the thyroid gland of a 4-months-old offspring of a ewe fed 240 μc. of I^{131} daily. Hematoxylin and eosin stain. × 115.



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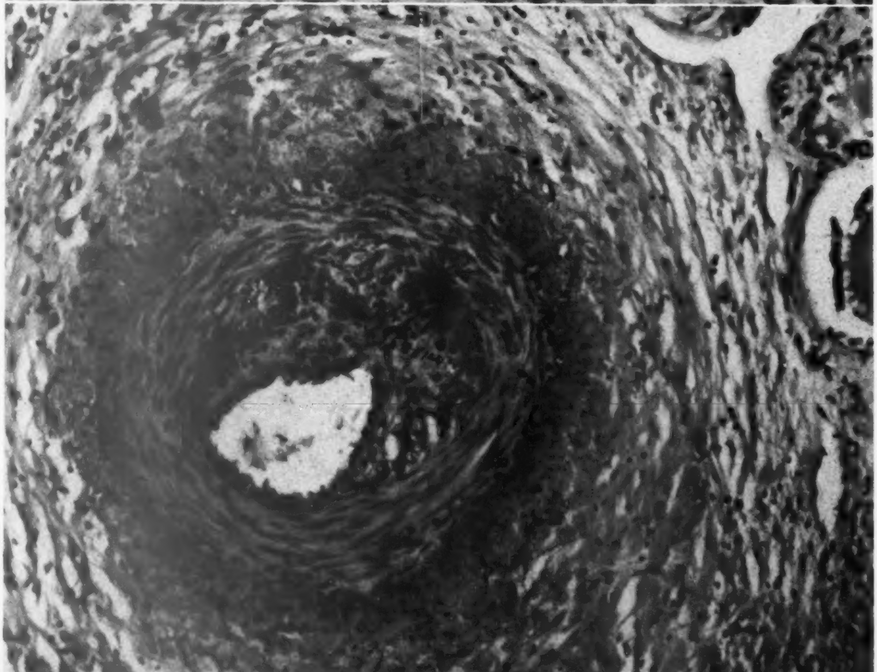

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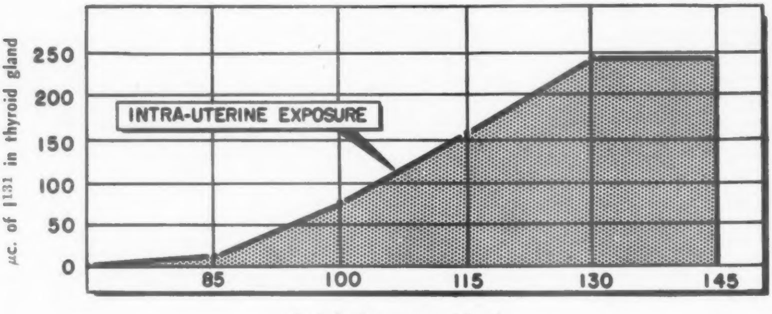


Chart 8

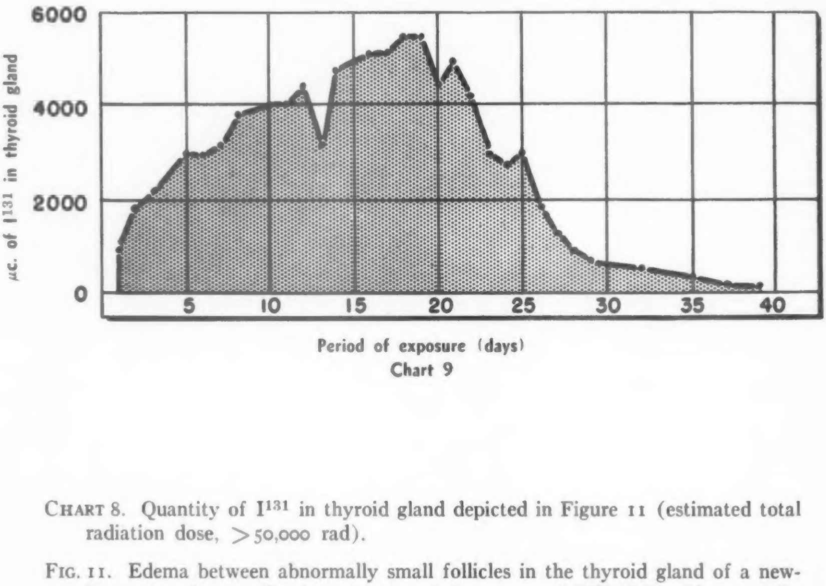


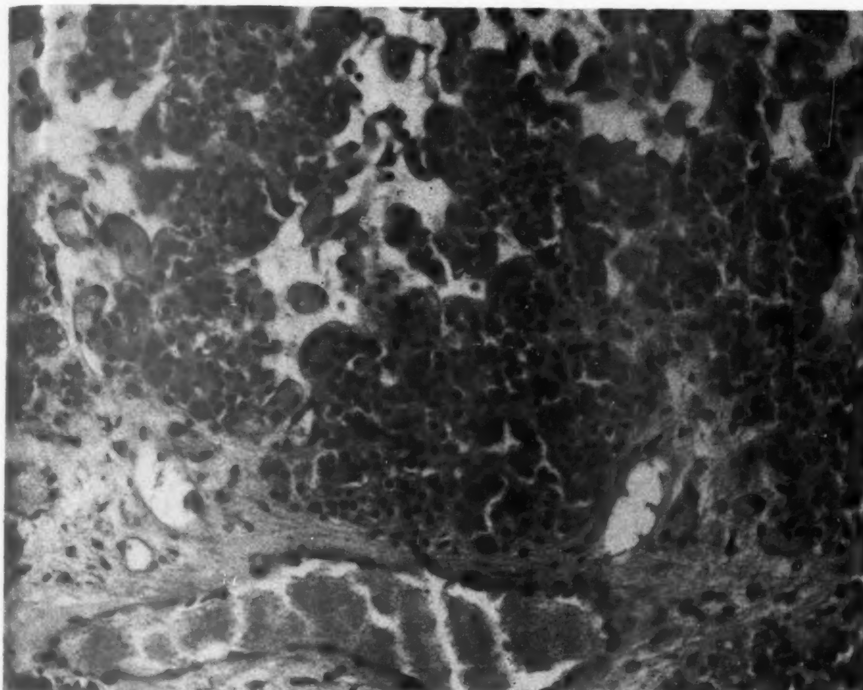
Chart 9

CHART 8. Quantity of I^{131} in thyroid gland depicted in Figure 11 (estimated total radiation dose, >50,000 rad).

FIG. 11. Edema between abnormally small follicles in the thyroid gland of a newborn second-year offspring of a ewe fed 240 μc. of I^{131} daily. Hematoxylin and eosin stain. × 150.

CHART 9. Quantity of I^{131} in thyroid gland depicted in Figure 12 (estimated total radiation dose, >100,000 rad).

FIG. 12. Minute remnants of follicles within a dense hyalinized scar in the thyroid gland of a ewe receiving 1,800 μc. of I^{131} per day for 420 days. Hematoxylin and eosin stain. × 185.



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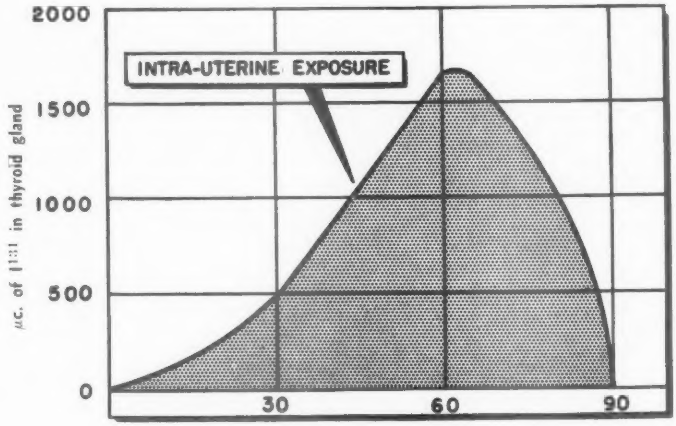
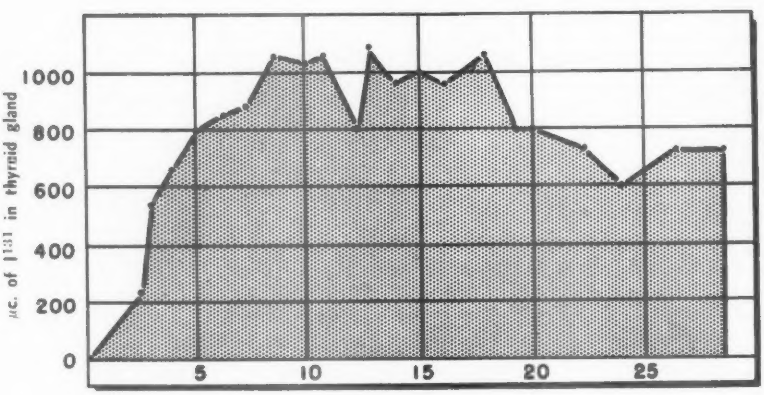


Period of exposure (days)
Chart 10



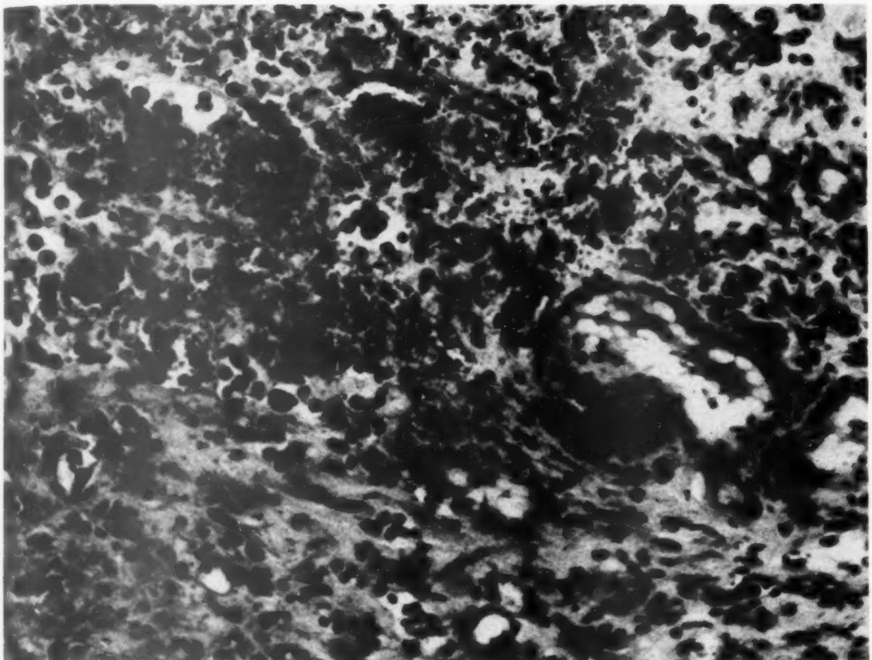
Period of exposure (days)
Chart 11

CHART 10. Quantity of I^{131} in thyroid gland depicted in Figure 13 (estimated total radiation dose, >100,000 rad).

FIG. 13. Central necrosis and hemorrhage in the thyroid gland of a newborn offspring of a ewe fed 1,800 μc. of I^{131} daily. Hematoxylin and eosin stain. × 310.

CHART 11. Quantity of I^{131} in thyroid gland depicted in Figure 14 (estimated total radiation dose, 22,000 rad).

FIG. 14. Large focus of predominantly lymphocytic infiltration between follicles in the thyroid gland of a yearling ram after 29 days of feeding of 480 μc. of I^{131} per day. Desquamated epithelial cells are present within lumina of follicles. Hematoxylin and eosin stain. × 320.



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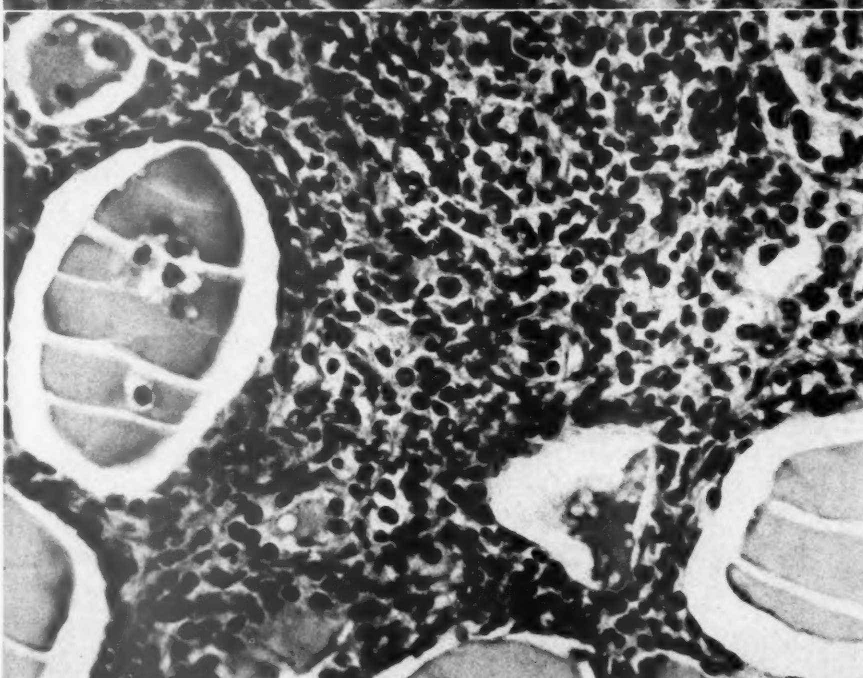

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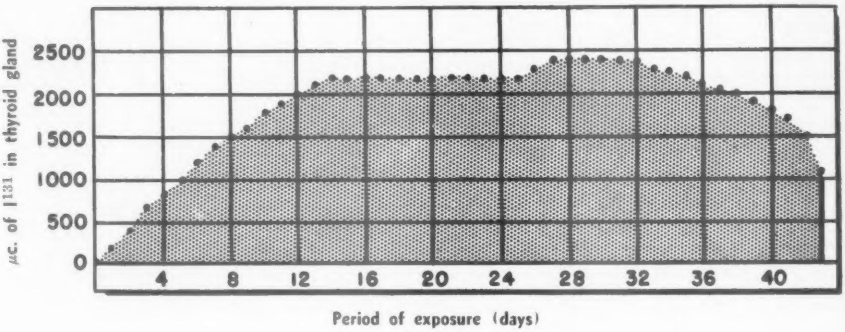


Chart 12

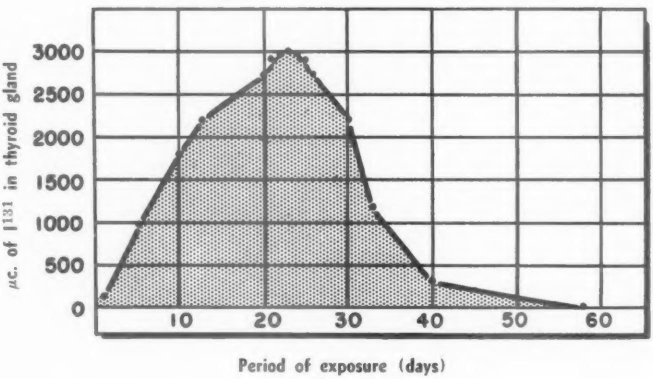


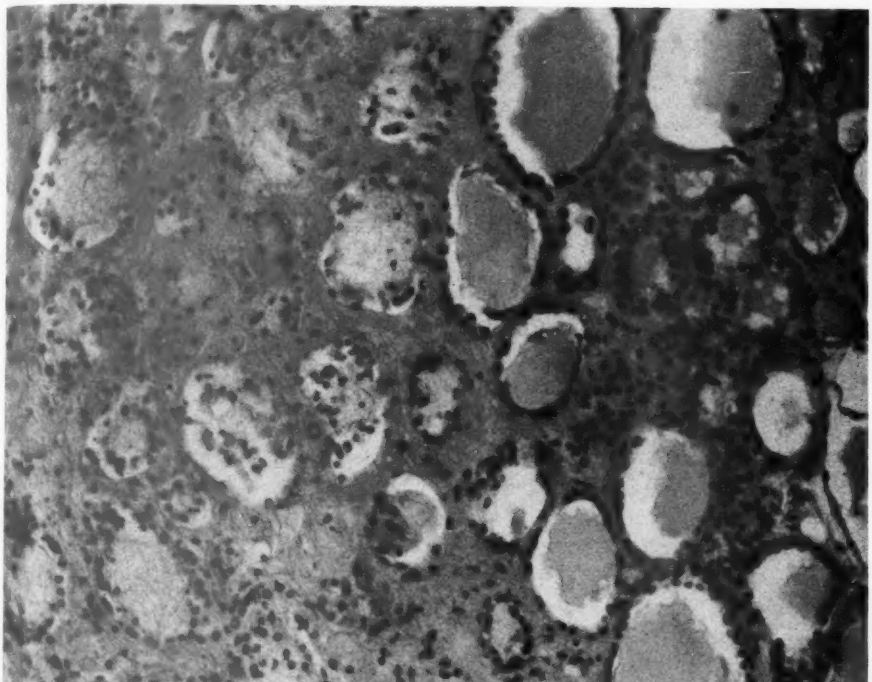
Chart 13

CHART 12. Quantity of I^{131} in thyroid gland depicted in Figure 15 (estimated total radiation dose, 80,000 rad).

FIG. 15. Infarct in the thyroid gland of a yearling ram fed 480 μc. of I^{131} per day for 43 days. Hematoxylin and eosin stain. × 160.

CHART 13. Quantity of I^{131} in thyroid gland depicted in Figure 16 (estimated total radiation dose, 70,000 rad).

FIG. 16. Giant cells within necrotic tissue in the thyroid gland of a yearling ram fed 480 μc. of I^{131} per day for 108 days. Hematoxylin and eosin stain. × 200.



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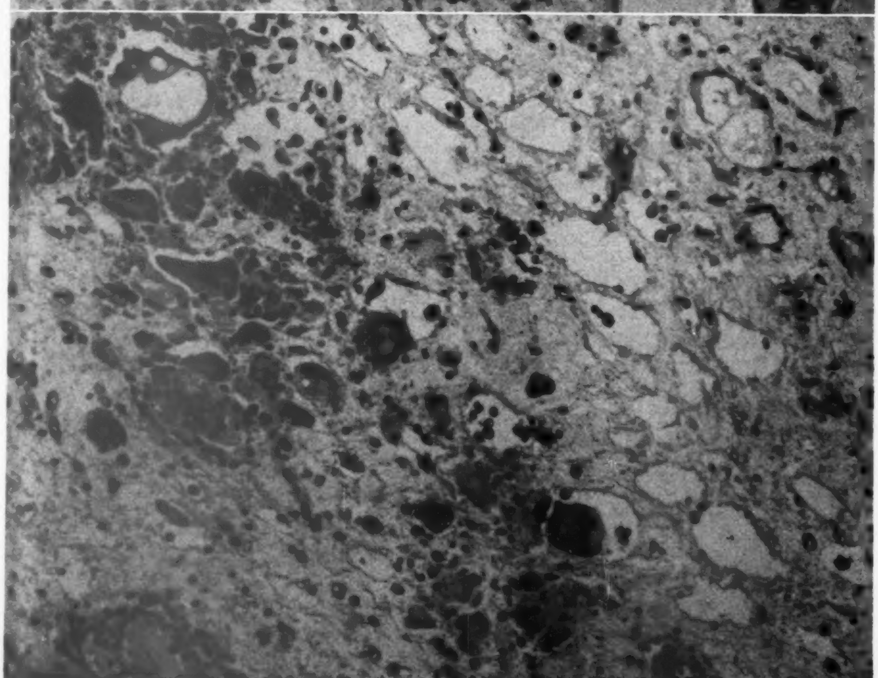

16

THE NATURE OF THE HYALINE THROMBI IN THROMBOTIC THROMBOCYTOPENIC PURPURA*

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Since the first definitive clinical and pathologic description of the syndrome of thrombotic thrombocytopenic purpura by Moschcowitz,[1] there has been debate concerning the nature of the hyaline thrombi in the dilated arterioles and capillaries. Partly because of the observation that destruction and agglutination of red blood cells occurred in transfusion reactions and partly because of the rapid fall of hemoglobin in this syndrome, Moschcowitz believed that the thrombi were composed of agglutinated or degenerated red blood cells. Baehr, Klemperer, and Schifrin[2] believed that hemoglobin, derived from erythrocytes, was not a component of the hyaline thrombi, because they lacked oxidase activity and free or bound iron. These authors demonstrated a granular appearance of the thrombi in sections stained by the Giemsa method, and this led them to believe that the thrombi were composed of agglutinated platelets. This belief was strengthened by the known decrease in circulating platelets associated with an adequate supply of megakaryocytes in the bone marrow. Their preparations stained by the Weigert technique showed little fibrin in the thrombi. Altschule[3] pointed out that capillary damage may antedate the thrombotic lesion, and a fibrinoid reaction in the vessel wall was noted by Carter.[4] Most authors concurred in the opinion that the thrombi were composed of platelets,[5-7] although Meacham *et al.*[8] pointed out that no direct proof of this existed. Orbison,[9] who clearly demonstrated aneurysmal formation in the lesions and found reactions for fibrin in the thrombi, expressed strong doubts that the agglutination of platelets and the development of occlusive thrombi could result in the formation of the aneurysms. He believed that the lesions fell into the group of so-called collagen diseases. This latter view was strengthened by the observation of similar renal glomerular lesions in lupus erythematosus disseminata.[10]

It has been shown by using fluorescein-labeled rabbit antisera against human fibrin, that fibrin may be present in lesions in which it would not have been detected by the use of standard histologic

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techniques. In this manner, fibrinoid was shown to be composed principally of fibrin.[11] It was shown also that Mallory's phosphotungstic acid-hematoxylin (PTAH), a Biebrich scarlet-aniline blue sequence, and Pearse's periodic acid-Schiff (PAS)-orange G-hematoxylin stain did not give positive reactions for fibrin with clots formed from purified fibrinogen and thrombin. The stains became positive for fibrin when the clotting mixture in which the fibrin was formed contained more than 1 gm. per cent of albumin or 0.05 gm. per cent of glutathione.[12] With this knowledge, 2 cases necropsied at the Children's Hospital, Boston, and having the features of thrombotic thrombocytopenic purpura were studied.

Methods

Rabbit antisera were prepared against human fibrin, albumin, gamma globulin, iron-binding globulin, and platelets. The rabbit antibodies were labeled with fluorescein isocyanate as described elsewhere.[13,14] Tissues were obtained at necropsy, frozen quickly in glass containers at −70° C., and stored at −30° C. Sections 4 μ thick were cut at −30° C., placed on glass slides, and then dried quickly in air. These sections were dipped briefly in 95 per cent alcohol to fasten the tissues firmly to the slides, then washed in 0.15 M NaCl in order to remove soluble proteins; they were then flooded with specific fluorescein-labeled antisera and again washed in 0.15 M NaCl to remove excess antibody. Duplicate sections were placed in 95 per cent alcohol for 20 minutes, dried, and allowed to react with labeled antisera without having been washed prior to this reaction. The sections were mounted in buffered glycerin and observed and photographed under the ultraviolet microscope. They were then washed, fixed in Zenker's acetic acid fluid, and stained by a Biebrich scarlet-aniline blue sequence.[15]

Tissue blocks were fixed also in Zenker's acetic acid fluid, embedded in paraffin, sectioned at 8 μ, and stained with hematoxylin and eosin, toluidine blue-eosin, Mallory's PTAH stain,[16] Biebrich scarlet-aniline blue stain,[15] and a modification of Pearse's PAS-orange G-hematoxylin sequence.[17]

Results

Case 1

A 22-months-old white female infant had been well except for eczema of the face and antecubital spaces until 2 months before her first and only admission, when she had diarrhea and chicken pox in succession. A 10-day interval of well-being then ensued. Eight days before death she vomited bile-stained material and developed a bloody diarrhea, which brought her to the hospital.

On admission 5 days before death, the infant was pallid, irritable, and had a slight

chronic eczematoid rash in the antecubital fossae and over the dorsum of one hand. The temperature was 99° F. and the pulse 144 per minute. The liver was felt 4 cm. below the right costal margin. The initial laboratory examinations revealed the hemoglobin to be 9 gm. per cent; leukocytosis, 17,000 per cmm.; platelets were decreased in number on the peripheral blood smear. A guaiac examination of the stools gave a 3 plus reaction. The blood culture revealed no growth. A urine sample obtained 2 days after admission had 100 to 250 erythrocytes per high-power field, granular casts, and a 1 plus reaction for albumin.

The patient improved for 2 days, then became lethargic, vomited, and developed strabismus and slight nuchal rigidity. Shortly thereafter she became comatose and had a convulsion. Hemoglobin was 5.9 gm. per cent; leukocyte count, 21,500 per cmm. with 45 per cent polymorphonuclear cells and 45 per cent lymphocytes. Blood platelets numbered 30,000 per cmm. and the reticulocyte count was 5 per cent. On this day cutaneous petechiae and a retinal hemorrhage were first observed. Four days after admission she passed several large tarry stools and went into peripheral vasomotor collapse; blood pressure was unobtainable. After restoration with a blood transfusion, blood studies revealed the platelet count to be 11,000 per cmm.; bleeding time, 14 minutes; prothrombin content, 55 per cent of normal; total bilirubin, 3.2 mg. per cent; serum non-protein nitrogen, 134 mg. per cent. A section of aspirated bone marrow showed no abnormalities of the capillaries or megakaryocytes. On the day of death the infant had further episodes of melena and finally went into peripheral vasomotor collapse with continued stupor. The pulse and respirations gradually decreased until death, 5 days after admission.

This child at necropsy had gross edema of the face and extremities, and free fluid was present in all serous cavities. There were ecchymoses about needle puncture marks and beneath the serosa of the large intestine, and petechial hemorrhages in the small intestine, liver capsule, and kidneys. The endothelial cells of the myocardial capillaries were swollen and proliferated, but no thrombi were seen, and no myocardial degeneration was evident. The lungs were atelectatic. There was marked edema of the alveoli. Occasional occlusive hyaline thrombi were present in the capillaries, precapillary arterioles, and small arteries. In rare instances subendothelial deposition of hyaline material was found. Megakaryocytes were frequent within the capillary loops. In the spleen, the endothelium of the penicillar arteries was swollen and proliferated, but contained no thrombi. The malpighian bodies showed a marked toxic reaction and the lymphoid tissue was greatly depleted. The intestine revealed small petechial hemorrhages in the lamina propria. The pancreas contained a few minor foci of necrosis. In the liver a widespread multilobular parenchymal necrobiosis was present; in most areas only the liver cells were affected and no inflammatory cells had entered. The kidneys were 20 per cent overweight and swollen. Microscopically, the glomeruli were swollen and often congested; fibrinoid alteration in the capillary walls was noted and frequent occlusive thrombi were seen within both the afferent arterioles and in the capillary tufts. Dilatation of the lumina of these structures was minimal. A few occlusive

thrombi gave a positive stain for fibrin by the PTAH, PAS-orange G-hematoxylin, or Weigert procedures, but the capillary endothelial basement membranes and the majority of thrombi did not (Fig. 2).

The thrombi fluoresced brilliantly in the ultraviolet light when stained with labeled antiserum against human fibrin (Figs. 1 and 3). This fluorescence filled many of the glomerular capillary lumina and often was seen in areas corresponding to the basement membranes of the glomerular tuft next to the capillary lumina. No fluorescence was demonstrable using labeled antiserum against human platelets (Fig. 4). There was necrosis and atrophy of the proximal convoluted tubules as well as of the distal tubules, loops of Henle, and collecting tubules. The kidney thus showed features of acute cortical necrosis. The bone marrow was hyperplastic with an increased number of megakaryocytes, many of whose nuclei appeared pyknotic. The brain was slightly overweight and, except for the swelling of the capillary endothelium and some perivascular hemorrhages, sections were histologically normal.

Case 2

An 11-year-old white boy was well until 1 week before his only hospital admission, when he noticed the spontaneous appearance of a black and blue mark on his calf. Four days prior to admission he complained of nausea, anorexia, and epigastric pain. A day later he was drowsy, and became listless. On the day of admission he became weak, dyspneic, and complained of numbness of the left side of his face and difficulty in speaking. The family physician noted purpuric areas on the lower extremities. Because of persistence of these signs the boy was admitted to the hospital, where he was found to be icteric, pale, and confused. Ecchymoses and petechiae were present on the extremities and trunk. The physical examination was not remarkable otherwise, though difficulty in speaking was obvious. The initial laboratory examinations revealed albumin and erythrocytic casts in the urine. The hemoglobin was 7 gm. per cent; hematocrit, 16 per cent; peripheral leukocyte count, 8,600 per cmm. The platelets numbered 9,000 per cmm. The blood cultures were negative. The patient's course in the hospital was marked by intermittent fever, waxing and waning of symptoms due to involvement of the central nervous system, recurrent crops of petechiae, and a continued low level of hemoglobin despite transfusions. The platelets never rose above 13,000 per cmm. One day before death he developed hematemesis and melena with rapidly falling hemoglobin and blood pressure. He died 24 days after the onset of his illness.

At necropsy, there was icterus, and widespread petechiae were found on the skin of the face, trunk, thighs, and oral mucous membranes. Ecchymoses involved the skin of the legs. The heart was twice the normal weight, and was enlarged and dilated; there were petechiae on the endocardium and pinpoint gray spots in the myocardium. Microscopically, numerous dilated small vessels containing thrombi were present throughout the epicardium and myocardium. The thrombi only rarely stained for fibrin with Mallory's PTAH stain. Many of the thrombi were endothelized and the vessels re-

canalized in numerous instances (Fig. 5). Endothelial proliferation was marked. Frequently, aneurysmal dilatation of small arterioles caused a compression of surrounding myocardial fibers. In a few scattered areas, fibrinoid change was evident in the perivascular zone. More frequently there was evidence of loss of myocardial cells due to ischemia. The masses accompanying these lesions showed intense fluorescence when stained with labeled antihuman fibrin serum (Figs. 6 and 7). In the majority of the lesions, the vessel wall did not show specific fluorescence; this was not unexpected, since the tails of the propagated clots would extend through many sections. In the lungs no thrombi were found, although large numbers of megakaryocytes were seen in the capillary lumina. The spleen was twice the normal weight, and the penicillar arteries contained large numbers of hyaline thrombi which gave specific fluorescence with labeled antiserum against fibrin. In the intestine similar vascular lesions were encountered in the lamina propria and submucosa, but rarely, if at all, in the muscularis. In the liver the hepatic arterioles contained hyaline thrombi, and these fluoresced with specific antifibrin antiserum (Figs. 8 and 9). Thrombotic lesions were present chiefly in the afferent arterioles of the kidney, in the glomerular capillaries, and in the small vessels of the medullary rays (Fig. 10). The occluded glomerular capillaries gave an intense fluorescence when stained with fluorescein-labeled antifibrin serum, and here fibrin deposition in the areas of fibrinoid alteration of thrombosed vessel walls was clearly demonstrated (Fig. 11). In the adrenal gland, the affected vessels were noted chiefly in the pericapsular, capsular, and subcapsular area; they did not appear in the sinusoids. No such lesions were present in the skin, testes, or thymus, but were numerous in the diaphragm and psoas muscle. They were seen also in the bone marrow, lymph nodes, and brain. In the brain they were more numerous in the paraventricular area of the third ventricle and in the neuronal ribbon of the cerebral cortex, both being areas of increased vascularity. No areas of brain destruction were evident, although nuclear changes in glial and nerve cells could be seen. The bone marrow was hypercellular and megakaryocytes were plentiful. The occlusive thrombi in the affected tissues, when stained with labeled antiserum against human platelets, gave no specific fluorescence.

Discussion

The short duration of life, restricted distribution of thrombotic lesions, lack of conspicuous endothelial proliferation, lack of aneurysmal dilatation in affected vessels, and rather extensive renal tubular

and hepatic necrosis in case 1 are features frequently seen in the generalized Shwartzman reaction produced in rabbits by repeated injections of meningococcus toxin.[18] In this patient, however, the fall in platelet levels, anemia, reticulocytosis, renal failure, and symptoms of damage to the central nervous system are consistent with the diagnosis of thrombotic thrombocytopenic purpura. The close relationship of the Moschcowitz syndrome to the generalized Shwartzman reaction has been pointed out.[10] The development of the Shwartzman reaction has been described also in epidemic diarrhea of the newborn.[19] The short duration of life after the onset of symptoms may be responsible for the failure of the full development of the picture of the Moschcowitz syndrome, especially the endothelial proliferation and organization of the thrombi.

Case 2 is an example of thrombotic thrombocytopenic purpura with a more protracted course, more typical clinical features, widespread thrombotic lesions with marked aneurysm formation, and organization and recanalization of the lesions.

In both cases, the specific fluorescence which occurred with labeled antisera against human fibrin and the absence of this fluorescence in proper controls indicated that fibrin or an immunochemically-reactive insoluble derivative of fibrinogen made up a large part of the thrombi. Some areas in the thrombi which did not show fluorescence appeared to be occupied by proliferated endothelial cells. It is suggested that the failure of the thrombi to react with labeled rabbit antihuman platelet serum indicated that immunochemically-reactive platelet material was not present in the lesion.

The intimate relationship of fibrin thrombi to an alteration in the vessel walls has been pointed out by numerous authors.[3,4,6] It was to be expected that many of the thrombi present in the tissues were propagated and hence the vessel wall would not necessarily show any alteration at the site of the section or even at the site of obvious dilatation. This was seen in the heart in case 2. Such dilatation beyond an obstructive lesion is reminiscent of the endarteritis seen in the lungs of patients in certain instances of pulmonary hypertension.[20] In many areas, the deposition of fibrin within the affected vessel wall was apparent and this was noted especially in the glomeruli of case 1, in which the outline of non-occluded capillary loops could be visualized (Fig. 3).

If, as seems likely, endothelial damage is the first event in the pathologic sequence leading to thrombosis, it is not unreasonable to

suggest that this change would directly, or indirectly via platelets, release thromboplastin for the conversion of fibrinogen to fibrin. Such a chain of events would also account for the subendothelial deposition of hyaline material, presumably fibrin, described by Gore.[6] We found great difficulty, however, in distinguishing intraluminal thrombi, over which endothelium had grown, from lesions with intact endothelium which showed a subendothelial deposition of fibrin. The frozen sections used for fluorescent studies were, unfortunately, not suitable for making such fine distinctions because of poor cytologic detail upon restaining.

Purified fibrinogen clotted in the presence of albumin concentrations of 1 gm. per cent or less gave negative histologic reactions for fibrin in artificial clot systems.[12] Albumin concentration, however, is probably not the only *in vivo* factor determining the staining reaction of fibrin. In interstitial fluid, the concentration of albumin normally is of the order of 0.6 to 1 gm. per cent and thus fibrin in lesions outside the vascular system may give negative reactions with the usual histologic methods.[11] In the fibrin thrombi of thrombotic thrombocytopenic purpura, however, even in the presence of albumin levels above 2.5 gm. per cent, the usual staining methods resulted in a negative reaction for fibrin. The reasons for this are not apparent at this time.

The observations made here give no indication of the essential nature of the Moschcowitz syndrome, nor do they explain other findings characteristic of this syndrome, such as thrombocytopenia or hemolytic anemia.

Summary

The thrombi in 2 cases of thrombotic thrombocytopenic purpura were found to react specifically with fluorescein-labeled rabbit antihuman fibrin antibodies, but were found to be negative or only occasionally and partially positive for fibrin when the tissues were fixed, embedded in paraffin, and stained by standard dye-staining procedures. These thrombi failed to react with fluorescein-labeled rabbit antihuman platelet antibodies.

It is concluded that the hyaline thrombi in thrombotic thrombocytopenic purpura are composed of a saline-insoluble derivative of fibrinogen, or fibrin.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

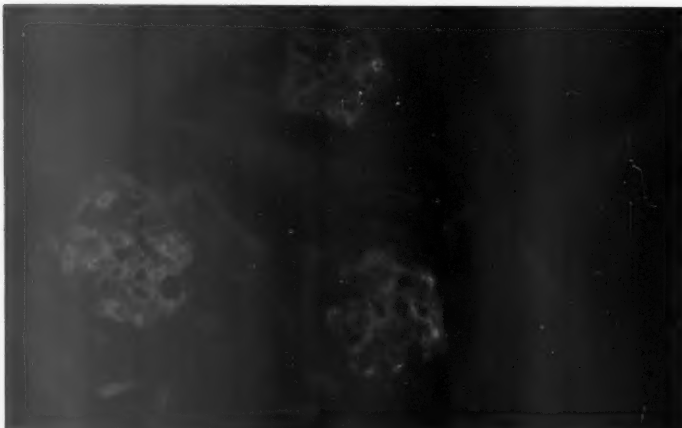
Figures 1, 3, 6, 7, 9, and 11 are photomicrographs of washed tissue sections stained with fluorescein-labeled rabbit antihuman fibrin serum as described in the text. Figure 4 is a photomicrograph of a washed tissue section stained with fluorescein-labeled rabbit antihuman platelet serum. The white areas represent specific fluorescence due to interaction of the labeled antibodies with the antigen in the section. The grayish areas are the result of a bluish autofluorescence of certain tissue structures. Under the ultraviolet microscope this bluish autofluorescence is easily distinguished from the greenish-yellow fluorescence of the fluorescein label.

FIG. 1. Case 1. Kidney. Three glomeruli showing specific fluorescence within and outlining the capillary loops. × 100.

FIG. 2. Case 1. Kidney. Glomerulus containing fibrin masses within the capillary lumina. Mallory's phosphotungstic acid hematoxylin (PTAH) stain. × 470.

FIG. 3. Case 1. Kidney. A glomerulus with specific fluorescence for fibrin in and occluding the capillary loops. × 210.

FIG. 4. Case 1. Kidney. A glomerulus stained with antiplatelet serum. No specific fluorescence is present; the light areas represent autofluorescence. × 210.



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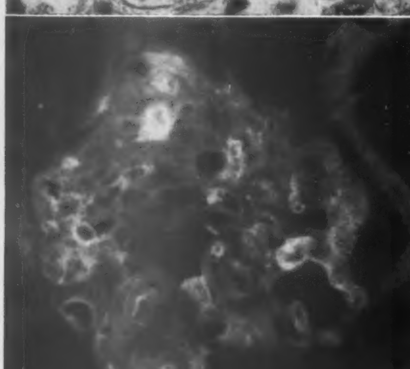

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FIG. 5. Case 2. Heart. Numerous dilated small vessels containing occlusive masses which are negative for fibrin. Mallory's PTAH stain. × 210.

FIG. 6. Case 2. Heart. Dilated small vessels with occlusive thrombi showing specific fluorescence. The dark areas in the thrombi are occupied by proliferated endothelial cells. × 100.

FIG. 7. Case 2. Heart. Specific fluorescence is present in the wall of a vessel. × 100.

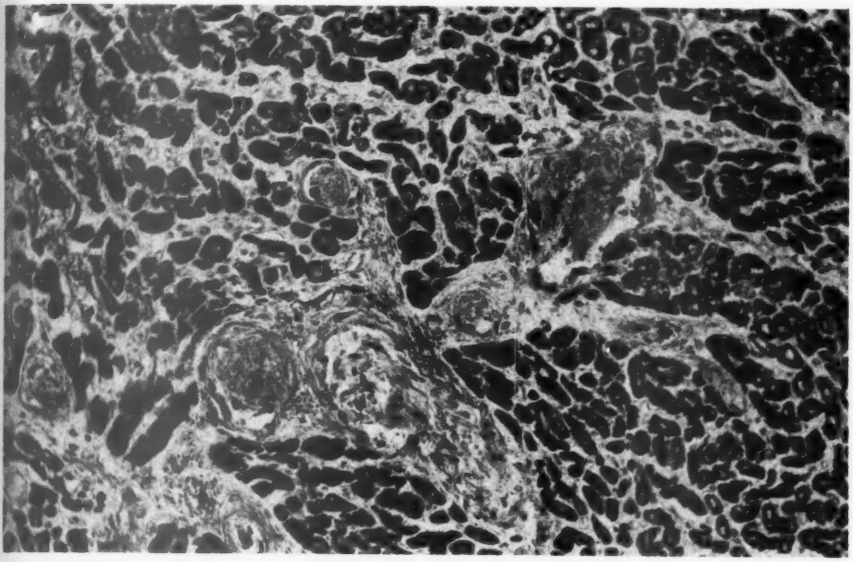

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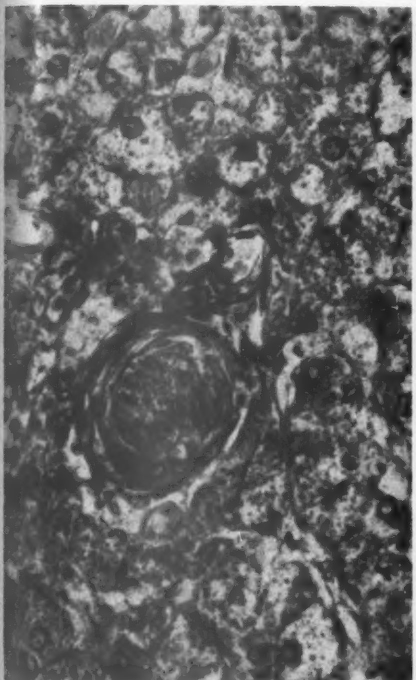
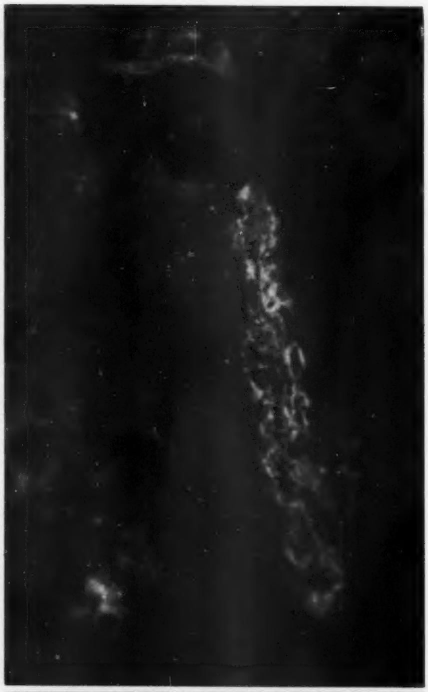
FIG. 8. Case 2. Liver. A dilated hepatic arteriole with an occlusive thrombus which is negative for fibrin with Mallory's PTAH stain. × 470.

FIG. 9. Case 2. Liver. A loosely arranged thrombus in an arteriole in a portal triad. × 210.

FIG. 10. Case 2. Kidney. A glomerulus showing occlusive masses of hyaline material in the glomerular capillaries. Hematoxylin and eosin stain. × 95.

FIG. 11. Case 2. Kidney. At upper left is a glomerulus showing specific fluorescence in occlusive masses in the capillaries; in three other areas the branches of a small artery contain material showing specific fluorescence. × 210.





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VARIATIONS IN THE STAINING CHARACTERISTICS OF HUMAN FIBRIN*

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It became apparent during an investigation of the nature of fibrinoid degeneration that fibrin in certain tissue lesions did not interact with histologic dyes in the generally accepted manner.[1] If the standard histologic methods for fibrin were used, fibrin could not be identified in these loci. This investigation, therefore, was undertaken to determine some of the conditions which influence the staining properties of fibrin.

Methods

In this study, series of fibrin clots were made; the composition of the medium in which the conversion of fibrinogen to fibrin occurred was controlled by the addition of various plasma proteins or cellular elements. In some instances, the electrolyte content of the medium was altered as well as the pH at which the clotting process took place. The clots were then sectioned and studied with a fluorescent antibody technique and by the use of some standard histologic staining methods for fibrin. The details follow.

Preparation of Fibrin Clots

Human fibrinogen and human thrombin, obtained by the low-temperature ethanol-water fractionation method,[2,3] were supplied by Dr. J. L. Oncley. The fibrinogen, SHI-232, was in the form of Fraction I[2] and, in accord with such crude fibrinogen fractions, 65 per cent of the protein in the preparation could be clotted with thrombin. The thrombin[3] contained 12.5 units per mg. of protein.[4] The fibrinogen and thrombin were dissolved in distilled water and saline solution, respectively, and prepared freshly for each experiment. The fibrinogen stock solution contained 35 mg. of fibrinogen per ml. and the thrombin was made to 10 units per ml.

Clots were formed in a chemical spot plate, the capacity of each depression being about 1 ml. of fluid. One tenth of 1 ml. of fibrinogen solution was added to several depressions and diluted with 0.8 ml. of

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the medium to be tested. After mixing, 0.1 ml. of the solution of thrombin was added, mixed, and the clot permitted to form. Thus the final mixture was 1.0 ml. in volume, and the final concentrations of fibrinogen and thrombin were 0.35 gm. per cent and 1 unit per ml., respectively. Clots were made in duplicate or triplicate for each variation in the composition of the clotting mixture. In the absence of additional buffer, the clotting process occurred at a pH of 6.5. A reaction time of 4 hours usually was allowed for completion of the clotting mechanism; reaction times from 1 to 48 hours revealed no significant differences in the staining properties of the fibrin.

In some experiments, plasma proteins were dissolved in the fluid used to dilute the fibrinogen prior to the addition of thrombin. Crystallized human serum albumin,[5] purified γ-globulin,[6] crystallized acid glycoprotein,[7] purified α- and β-lipoproteins,[6] crude iron-binding globulin,[8] and cold-insoluble globulin were used in these studies. The concentrations of these proteins were varied over ranges which extended to levels far in excess of their normal concentrations in plasma. The selected protein was dissolved in 0.15 M NaCl and this was used as the diluting fluid for fibrinogen; the *final* concentrations of these proteins in the clotting mixture are presented in Tables I to IV.

In other experiments, glutathione, cysteine, methionine, or ethylenediamine-tetra-acetic acid (EDTA) was dissolved in 0.15 M saline solution and used as a diluting fluid. The final concentrations in the clotting mixtures are given in Tables I to IV.

Platelets from 5 cc. of fresh normal blood were separated, suspended in 0.15 M NaCl, and this was used as the diluent for an individual clotting mixture. Separated platelets from 100 cc. of blood, kept at 4° C. for 6 months in gelatin, were used in a similar manner.

Instead of using 0.15 M NaCl as a testing medium or diluting fluid for simple fibrinogen-thrombin interaction, phosphate buffer at a pH of 7.4 was substituted ($\Gamma/2$ phosphate $= 0.05$, $\Gamma/2$ NaCl $=$ 0.10) in some cases, or combinations of 0.1 M potassium carbonate (K_2CO_3) and 0.1 M calcium chloride ($CaCl_2$) were used.

Fresh plasma was collected without anticoagulants in lucite tubes and was then permitted to clot spontaneously. In several cases, the plasma was diluted 1:5 with 0.15 M saline solution and the clotting process initiated with thrombin. Fresh plasma collected in 0.1 volumes of 2.5 gm. per cent of sodium citrate was used also, thrombin being employed as the clotting agent.

Since spontaneous syneresis of fibrin is a normal sequence in the

natural clotting process and since the clots formed in this study did not contract spontaneously in the absence of plasma, free fluid was absorbed from representative clots with filter paper. In a few instances, the fibrin from the clotting mixtures was wound on a glass rod as the clot developed and thus very dense fibrin strands were obtained.

Preparation and Staining of Sections

In some experiments, the clots were frozen rapidly in a small bottle pre-cooled to –70°C. and sectioned at –30°C. in a special cryostat.[9] The sections were dried on slides and then dipped in 95 per cent alcohol for 30 seconds to fix the tissues to the slides. Occluded soluble proteins[1,10] were washed from the sections with several changes of 0.15 M NaCl. The sections were then flooded with fluorescein-labeled rabbit antiserum against human fibrin, and, after 15 minutes, the excess labeled antiserum was removed by washing with 0.15 M NaCl. After mounting in buffered glycerin, the sections were examined for specific fluorescence under the ultraviolet light microscope and photomicrographs taken; the coverslips were removed and the sections were fixed in Zenker's acetic acid fluid and restained with a Biebrich scarlet-aniline blue sequence. This procedure is described in detail elsewhere.[1,9,11] As controls for the specificity of this method, replicate sections were reacted with fluorescein-labeled rabbit anti-fibrin that had been adsorbed with washed human fibrin or human fibrinogen. Additional controls were prepared by using unlabeled rabbit anti-fibrin on the sections prior to flooding with labeled antiserum; specificity was indicated by little or no fluorescence in the sections. The anti-fibrin antisera were adsorbed with normal human serum prior to use, as a precaution to ensure the specificity of the antisera for fibrin or fibrinogen; it must be noted that anti-fibrin antisera did cross-react with fibrinogen.[11,12] Other controls were obtained by staining with labeled rabbit anti-human albumin and anti-human γ-globulin.

Replicates of the clots studied by the fluorescent antibody method and all other clots were fixed in Zenker's acetic acid fluid and embedded in paraffin. Replicate sections were cut at 8 μ and stained with Mallory's phosphotungstic acid-hematoxylin (PTAH) method,[13] a Biebrich scarlet-aniline blue sequence,[14] Pearse's periodic acid-leukofuchsin (PAS)-hematoxylin-orange G stain,[15] and Weigert's fibrin stain. It must be noted that when the clots were fixed, all the non-fibrinous protein occluded in the clots and capable of interacting with dyes was still present. Thus, when protein fractions other than

thrombin and fibrinogen were present in the clotting mixtures, sections cut from such fixed clots differed from frozen sections of the unfixed clots that were washed. In the latter instance, occluded proteins were removed from the section.

Spectrophotometric Absorption Curves of Stained Sections

The absorption spectra of stained sections of the fibrin clots from 390 to 710 mμ were determined in a Beckman DU spectrophotometer. The slide which held the section to be studied was mounted with tape on a rectangular piece of cardboard, the fibrin section being placed directly over a small perforation in the board. The cardboard thus served as a mask and permitted only the light transmitted through the section to hit the photocell. An unstained section mounted in the same way was used as the blank.

The dye-binding capacities of the fibrin clots for some of the individual dyes used in the staining methods were estimated for pH levels from 2.5 to 8.0 by determining the optical densities of the stained sections in the spectrophotometer; the cardboard mask was used as described previously. The binding ability of methylene blue by these clots was studied similarly. McIlvaine's buffers, 0.1 M, were used[16] and the dye concentrations were 5×10^{-4} M throughout. Spectrophotometric absorption curves were determined for these sections as well as for the pure dyes in buffer solutions over the same pH range. Appropriate blanks were used.

RESULTS

All of the fibrin clots studied with the fluorescent antibody method showed specific fluorescence for fibrin under the ultraviolet light microscope (Figs. 1 to 4). The interaction of fibrin with its specific labeled antibody did not appear to be influenced by the composition of the medium in which the clotting process occurred. When the clot consisted of very dense fibrin strands or masses, there was a tendency for the edges of the fibrin to fluoresce more brightly than the central portions (Figs. 2 and 4).

The reactions which were obtained with the fibrin clots fixed in Zenker's acetic acid fluid and stained with the various histologic dyes were of three types: (1) a positive reaction for the presence of fibrin indicated by a deep blue coloration with Mallory's PTAH stain (Figs. 5, 7, and 8), a deep red-orange with Biebrich scarlet-aniline blue stain (Figs. 5 and 6), an orange yellow with Pearse's PAS-hematoxylin-orange-G sequence (Fig. 5C); (2) a negative reaction for fibrin

TABLE I

Fibrin Clots Giving Positive Staining Reactions (Composition of the Clotting Mixtures)

Form of clotting protein	Clotting agent	Diluent	Remarks
A. Coarse fibrin network			
Plasma	None	None	After syneresis or rolling strands on glass rod
Plasma and fibrinogen	None	Albumin, 5 gm.%	
Fibrinogen	Thrombin	Serum and platelets	Fluid absorbed from clot
B. Crystalline fibrin network			
Plasma	None	None	No syneresis or minimal syneresis permitted
Citrated plasma	Thrombin		
Fibrinogen	Thrombin	Albumin, 5 gm.%	
Fibrinogen	Thrombin	Serum	
Fibrinogen	Thrombin	Glutathione, > 0.05 gm.%	

shown by a yellow-red (Figs. 5F and 10), deep blue (Fig. 5G), and a reddish coloration (Fig. 5H) by these respective staining methods; and (3) an intermediate reaction for fibrin in which the color of the fibrin clot could not be considered either positive or negative. The stains were uniform in their reactivity with any given fibrin clot and thus if one stain gave a positive or negative reaction, the others reacted similarly. Weigert's fibrin stain, however, gave a uniform blue stain in all of the clots studied irrespective of the reactions of the other histologic dyes.*

The clots which gave a positive histologic reaction for fibrin with the dyes could be sub-divided into two groups: (A) those in which a coarse fibrillar pattern was predominant (Fig. 7) with numerous granules in the interstices, which also gave a positive reaction, and (B) those in which the fibers were fine (Fig. 8) with short rodlets that were uniform in caliber. Examples of the composition of the clotting mixtures which resulted in these forms are given in Table I. It is noted that all of the fibrin clots that gave a positive reaction were associated

* In the color plate the deep blues and yellows in the PTAH and Pearse stains have purple and green hues, respectively.

with the presence of albumin or glutathione in the clotting mixtures. Nineteen combinations other than those listed were studied and it was found that either albumin had to be present in a final concentration greater than 1 gm. per cent or glutathione in a concentration greater than 0.05 gm. per cent for a dye-positive fibrin clot to result. In general, removal of excess fluid from the fibrin clot by spontaneous syneresis, by absorption, by winding the fibrin on a glass rod, or otherwise distorting the clot mechanically prior to fixation, resulted in the coarse trabecular pattern (Figs. 2 and 4). The granules in the clots of the coarse type undoubtedly represented occluded proteins. Albumin or plasma dropped into Zenker's acetic acid fluid, embedded and sectioned, revealed granules of the same type as those that reacted positively with the fibrin stains.

Some of the fibrin clots that gave a negative reaction for fibrin with the dyes are listed in Table II and are shown in Figures 5 (F

TABLE II

*Fibrin Clots Giving Negative Staining Reactions**

Diluent	Remarks
0.15 M NaCl	With or without absorption of fluid from clot
Fresh platelets	
Albumin, 0.3 gm.%	
Cold insoluble globulin, 5 gm.%	
β-Lipoprotein, 1 gm.%	
Acid glycoprotein, 0.65 gm.%	
$CaCl_2$, K_2CO_3	With or without absorption of fluid from clot
Glutathione, $<$0.005 gm.%	
Cysteine, $>$0.2 gm.%	
Methionine, $>$0.2 gm.%	

* All clotting mixtures contained fibrinogen and thrombin.

to H) and 10. The presence of albumin in a concentration below 1 gm. per cent, 5 gm. per cent of cold insoluble globulin, 5 gm. per cent of β-lipoprotein, 0.65 gm. per cent of acid glycoprotein, platelets, cysteine up to a concentration of 0.2 gm. per cent, methionine up to a concentration of 0.2 gm. per cent or of glutathione up to a concentration of 0.005 gm. per cent failed to induce a positive reaction for fibrin with the stains. Mechanical distortion of the clot did not alter the staining characteristics. All of these fibrin clots gave a positive reaction with the PAS when the counterstains, orange-G and hematoxylin, were omitted.

Sections from some of the fibrin clots showed focal areas of definitely positive and negative reactions for fibrin, as noted in Table III

and Figures 5D, 5E, and 9. Some of these preparations contained serum at a final dilution of 1:5 or 1:10, or albumin at a final concentration of 1 gm. per cent. It is conceivable that in these instances the positive areas resulted from inadequate mixing of the clotting mixture prior to the addition of thrombin. There were rare instances, however, in which small focal areas gave positive reactions with the Biebrich and Mallory stains when the clotting mixture was fibrinogen, saline solution, and thrombin or when fibrinogen was fixed directly in Zenker's acetic acid fluid. An explanation for this was not evident.

TABLE III

*Unusual Fibrin Clots with Focal Positive and Negative Areas**

Diluent	Remarks
0.15 M NaCl	Fluid absorbed from clot
Serum 1:5	Fluid absorbed from clot
Albumin, 1 gm.%	
Glutathione, 0.01 or 0.02 gm.%	

* All clotting mixtures contained fibrinogen and thrombin.

The composition of some of the clotting mixtures which resulted in fibrin clots representative of the third type of reaction, the intermediate type, are listed in Table IV. This reaction was characterized by an equivocal degree of staining, the over-all color being intermediate between that of the positive and negative reactions for Mallory's PTAH stain, the Biebrich stain, and Pearse's sequence (Fig. 5J). The resulting colors were such that the reactions would not ordinarily have been considered positive for fibrin when observed in tissue sections. It is noted that some of the clotting mixtures contained albumin concentrations of about 1 gm. per cent added either as the pure protein or as diluted plasma; it is emphasized that 25 per cent of the iron-binding globulin preparation was serum albumin. In many of these fibrin clots, a fine crystalline pattern of fibrils gave a negative reaction, but in the interstices there were irregular masses of occluded protein which gave a positive reaction. The fibrin clot

TABLE IV

*Fibrin Clots Giving Intermediate Staining Reactions**

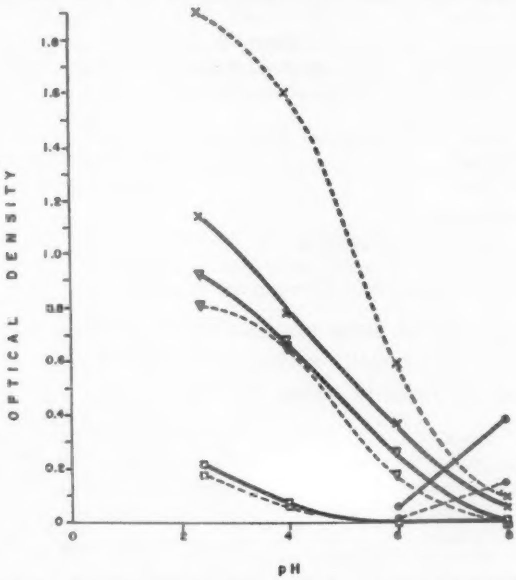
Diluent
γ-Globulin, 5 gm.%
Iron-binding globulin, 5 gm.%
Albumin, 1 gm.%
α-Lipoproteins, 1 gm.%
EDTA
None†

* All clotting mixtures contained fibrinogen and thrombin except where noted.

† Instead of fibrinogen, the clotting component was in the form of 1:10 diluted plasma and either clotted spontaneously or by the addition of thrombin.

formed in the presence of 0.2 gm. per cent of EDTA was singular in that it gave a strongly positive reaction with Mallory's PTAH method and Pearse's procedure, but gave a negative reaction with Biebrich scarlet-aniline blue stain.

To obtain additional objective information regarding the interaction of dyes with fibrin, sections from a positive fibrin clot formed from fibrinogen, thrombin, and 5 gm. per cent of albumin and a negative fibrin clot formed from fibrinogen and thrombin were stained

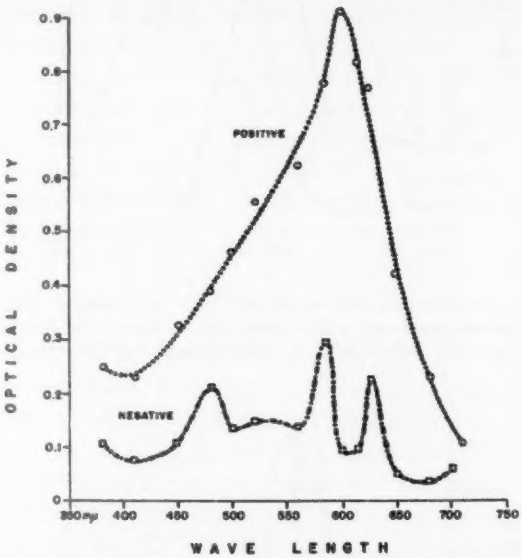


Text-figure 1. The dye-binding capacities of fibrin clots fixed in Zenker's acetic acid fluid, embedded in paraffin, and stained with methylene blue (dots), aniline blue (x's), Biebrich scarlet (triangles), and orange-G (squares). Positive reactions for the respective dyes are represented by solid lines; negative reactions by interrupted lines.

individually with each of the following dyes: aniline blue, orange-G, Biebrich scarlet, and methylene blue. The staining procedures were done in 0.1 M McIlvaines' buffers at a pH of 2.5, 4, 6, and 8 for 20 hours at room temperature with a dye concentration of 5×10^{-4} M. The results of spectrophotometric determinations are indicated in Text-figure 1. It is noted that positively-reacting fibrin sections had a slightly greater affinity for Biebrich scarlet and orange-G and less affinity for aniline blue at each pH than did the fibrin sections that gave a negative reaction with the Biebrich or Mallory stains. This is in accord with the visual findings. The positive-fibrin sections had a

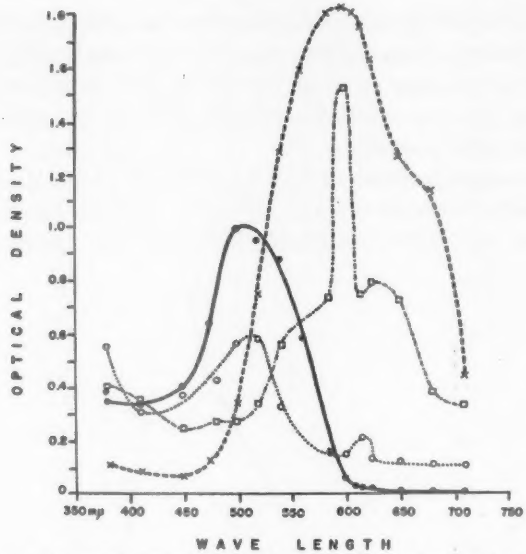
slightly greater affinity for methylene blue than did the negative-fibrin sections. Since the dye-binding capacities of hematoxylin and leukofuchsin were not dependent on the pH to any significant degree in the staining procedures which were used, interactions with these dyes were not done individually.

Absorption spectra were obtained for sections from the fibrin clots stained with Mallory's PTAH, Biebrich scarlet-aniline blue, and Pearse's sequence. The spectra are shown in Text-figures 2 to 4. It

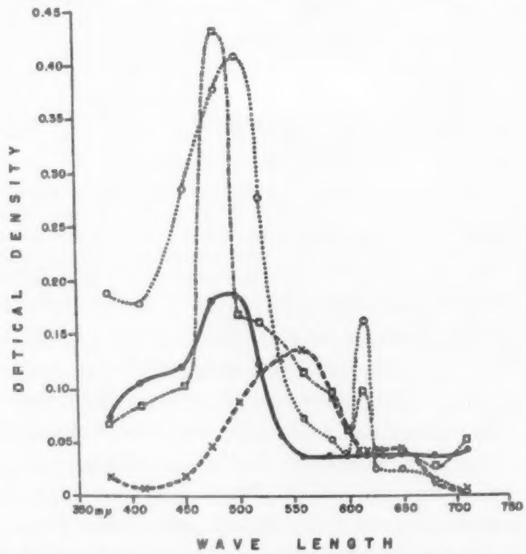


Text-figure 2. Absorption curves of sections from representative positive and negative fibrin clots stained with Mallory's phosphotungstic acid-hematoxylin. The positive clot, though stained a deep blue, shows absorption over the whole visible spectrum.

is seen that the peaks of absorption for each of the individual dyes were still evident in both the positively and negatively reacting fibrin clots, although no evidence of the submerged dye could be observed visually in the latter. Absorption spectra of the pure dyes in buffer solutions over the same pH range as was used in the dye-binding experiments were obtained also; no significant changes were noted for Biebrich scarlet or orange-G, but at an alkaline pH aniline blue was converted to the leuko base. The addition of albumin to 1 gm. per cent concentration did not significantly alter these curves. It would appear that the interaction of these dyes with fibrin (and albumin) did not bind groups that would alter their spectral characteristics. Hematoxylin and basic fuchsin were not investigated.



Text-figure 3. Absorption curves of sections from representative fibrin clots stained with Biebrich scarlet (dots) and aniline blue (x's); sections from positive (circles) and negative (squares) clots stained with the Biebrich scarlet-aniline blue combination are shown also.



Text-figure 4. Absorption curves of sections from representative fibrin clots stained with orange-G (dots) and basic fuchsin (x's). Sections from positive (circles) and negative (squares) clots were stained by Pearse's periodic acid-leukofuchsin-orange G-hematoxylin method; the peak at 610 mμ in the latter two curves was due to hematoxylin.

Discussion

It should be emphasized that the sections from some of the fibrin clots fixed in Zenker's acetic acid fluid contained occluded proteins and that some of these proteins, such as albumin, gave positive reactions with the staining methods. This was clearly exemplified by the fibrin clots which gave intermediate reactions. Hence considerable care was exercised to insure that, in the sections which gave a positive reaction, the fibrin fibrils were stained.

It was noted that the fibrin clots which reacted in a positive manner with the conventional histologic dyes were those formed from clotting mixtures containing either albumin in a final concentration greater than 1 gm. per cent or glutathione in a final concentration greater than 0.05 gm. per cent or, in one instance, tremendous numbers of preserved platelets. It is possible, in the latter case, that components in the platelets reached significant proportions because of the large numbers of platelets used.

The increased binding of both acidic and basic dyes, and the decrease in binding of aniline blue, an acidic dye, by the positively reacting fibrin clots as compared to the negatively reacting clots suggest a dissimilarity in the internal bonding structures of the differently reacting forms of fibrin. It would seem, therefore, that albumin and glutathione can influence the nature or number of the cross-linking bonds in fibrin. This is in accord with the findings of others that there are two forms of fibrin, one soluble and the other insoluble in urea.[17] Calcium plus a non-dialyzable fraction of plasma, the Laki-Lorand factor, are required for the formation of the urea-insoluble form of fibrin from fibrinogen. The same effect, however, may be reproduced by either albumin[18] or glutathione[17] in the concentrations used in this study to produce positively reacting fibrin clots. The urea-insoluble fibrin is said to have the cross-linkages present in the urea-soluble fibrin plus additional stronger bonds.[17] A similar situation would appear to exist in respect to the positively reacting fibrin clots, since in these there was an increase in the number of available groups that reacted with orange-G, Biebrich scarlet and methylene blue and a decrease in the groups that were available for binding aniline blue.

A concentration of albumin greater than roughly 1 gm. per cent was necessary for the formation of fibrin that would react positively with the selected stains. It is important to note that the normal concentration of albumin in the interstitial fluid of muscle and skin in

man is about 0.6 to 1.0 gm. per cent.[19] Thus, it is conceivable that fibrin in interstitial spaces might, under certain conditions, give a negative reaction with these stains; this has been found to be the case.[1] In certain effusions, however, due to inflammatory conditions or to increases in venous pressure, the concentration of albumin may reach 2.5 to 3.0 gm. per cent; hence, these situations would be conducive to the formation of fibrin that reacts positively with the stains; this also has been found to occur *in vivo*.[1]

The principles involved in Mallory's PTAH procedure are much more complex than simple acid-base interactions, but it is of interest that the staining reactions of this combination of dyes followed those of Biebrich scarlet-aniline blue and Pearse's orange-G sequence. The periodic acid-leukofuchsin reaction is not determined by acidic or basic groups and was positive in all the fibrin clots studied; in the fibrin clots formed with albumin in the clotting mixtures, however, the orange-G counterstain visually submerged the pink stain resulting from the former reaction. The hematoxylin of Pearse's procedure had no rôle in the determination of a positive or negative reaction for fibrin, but by spectroscopic analysis even this dye was bound by fibrin. Only in fibrin clots formed in the presence of EDTA was there any serious discrepancy found between Biebrich scarlet-aniline blue, Pearse's method, and Mallory's method.

Summary

Fibrin clots were prepared from purified fibrinogen and thrombin, either alone or with additional plasma proteins. Sections from these clots, with the exception of those prepared in the presence of concentrations of albumin greater than 1 gm. per cent, were shown to react specifically with fluorescein-labeled rabbit anti-human fibrin antibodies, but were found to react in a manner generally regarded as negative when they were fixed, embedded in paraffin, sectioned, and stained by three independent standard histologic procedures: Mallory's phosphotungstic acid-hematoxylin, Biebrich scarlet-aniline blue, and Pearse's periodic acid-leukofuchsin-hematoxylin-orange G.

When fibrin clots were formed in the presence of albumin at a concentration greater than 1 gm. per cent or glutathione at a concentration greater than 0.05 gm. per cent, the resulting sections reacted positively for fibrin by the standard staining techniques. The fluorescent antibody method indicated the presence of fibrin in such clots.

Dye-binding data and additional information are presented to sug-

gest that the fibrin reacting positively with dyes and the fibrin reacting negatively with the same dyes represent two forms of the material that differ in their structural bonds or cross-linkages. The evidence suggests that the positively reacting form of fibrin is related to the urea-insoluble form of fibrin and that the negatively reacting form is related to the urea-soluble form of fibrin.

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LEGENDS FOR FIGURES

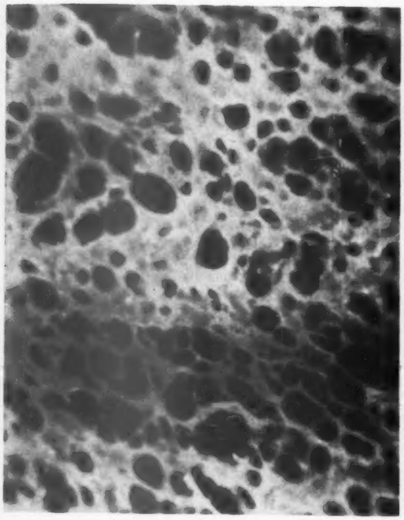
Figures 1 to 4 are photomicrographs of unfixed sections of fibrin clots stained with fluorescein-labeled rabbit antihuman fibrin.

FIG. 1. Clot formed from a mixture containing purified fibrinogen, albumin, and thrombin in a spot-plate and frozen without removal of fluid from the interstices of the clot. × 100.

FIG. 2. Clot formed from purified fibrinogen and thrombin; fibrin strands were collected on a moving glass rod as the clotting process occurred. × 100.

FIG. 3. Same clot as shown in Figure 1. × 400.

FIG. 4. Same clot as shown in Figure 2. × 400.



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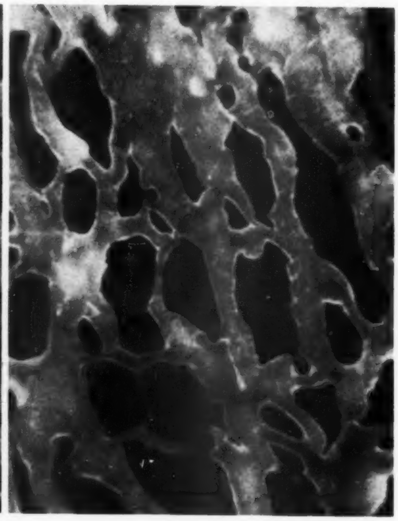

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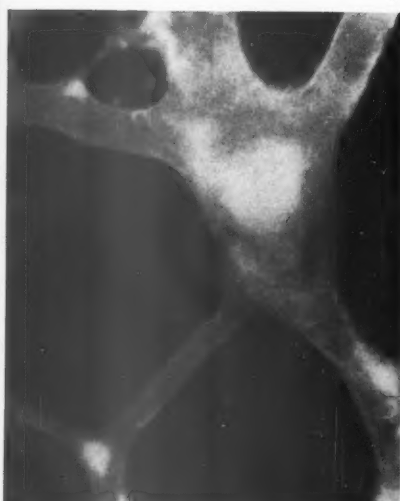

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FIG. 5. Photograph of slides on which were mounted stained sections from representative fibrin clots. Fibrin that gave a positive reaction is shown on slides A to C; fibrin with a negative staining reaction is shown on slides F to H; an example of the focal-staining fibrin is seen on slides D and E; an example of the intermediate staining reaction is seen on slides I and J.

A, D, F, and I: stained by Mallory's phosphotungstic acid-hematoxylin (PTAH) method.

B, E, G, and J: stained by Biebrich scarlet-aniline blue sequence.

C and H: stained by Pearse's periodic acid-leukofuchsin-hematoxylin-orange-G sequence.

FIG. 6. Sections from a fibrin clot of the same composition as that shown in Figure 1, but stained with Biebrich scarlet-aniline blue and showing a positive reaction. × 100.

FIG. 7. Section from a fibrin clot showing a coarse positive pattern. Mallory's PTAH stain. × 1000.

FIG. 8. Section from a fibrin clot showing a fine positive pattern. Mallory's PTAH stain. × 1000.

FIG. 9. Section from a fibrin clot showing mixed negative and positive staining. Mallory's PTAH stain. × 1000.

FIG. 10. Section from a fibrin clot showing negative staining reaction. Mallory's PTAH stain. × 1000.

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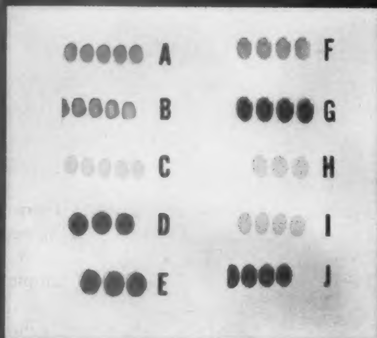

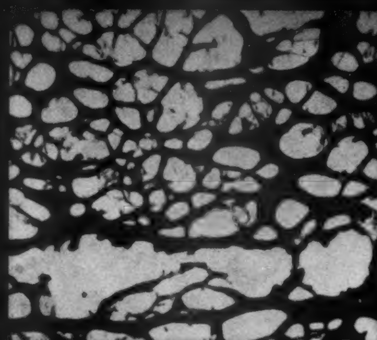

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A DIFFERENTIATION OF CERTAIN TYPES OF FIBRINOID AND HYALIN*

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The term fibrinoid[1] has been employed to describe a type of change occurring within connective tissues and blood vessels. Within connective tissues it has been observed in a variety of states, such as rheumatic fever,[2] rheumatoid arthritis,[3] disseminated lupus erythematosus,[4] as well as in the bases of benign peptic ulcers[1] and in certain lesions of the placenta.[1] In blood vessels fibrinoid has been encountered in malignant hypertensive cardiovascular disease,[5,6] eclampsia,[7-9] periarteritis nodosa,[10,11] thrombotic thrombocytopenic purpura,[12] the Shwartzman phenomenon,[13] and in certain experimental states resembling malignant hypertension of man.[14]

Hyalin, on the other hand, refers to a clear, homogeneous, structureless material with the normal prototype of hyaline cartilage.[15] It also involves connective tissues and blood vessels, and notably the small arteries and arterioles.

We wish to consider a differentiation of two types of fibrinoid and two types of hyalin. These are the fibrinoid of connective tissues and blood vessels and the hyalin of connective tissues and blood vessels.

Materials and Methods

For the purpose of studying connective tissue fibrinoid, two subcutaneous nodules of rheumatoid arthritis and one acute gastric ulcer were selected. For vascular fibrinoid, the kidneys from 11 cases of malignant hypertension of man which showed the typical acute arteriolar necrosis of that disease were selected.

Connective tissue hyalin was studied as exhibited by hyalinized connective tissue from an old subcutaneous scar, one meningioma, two keloids, three relatively inactive cases of scleroderma, and one uterine leiomyoma with marked hyalinization. For vascular hyalin it was elected to study the hyalinized renal arterioles encountered in 6 cases of diabetic nodular intercapillary glomerulosclerosis.

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TABLE I

*Histochemical Reactions for Types of Fibrinoid and Hyalin**

Materials†	Staining procedures and reactions															
	Mallory's stain	Masson's stain	Oil red O	Nile blue sulfate	Sudan black B	Schultz reaction	Congo red	Xanthoproteic reaction	Indole	Free carbonyl groups	Millon reaction	Calcium	PAS, frozen	PAS, permanent	Protein-bound SH	Potassium
Fibrin	Red	Red	o	o	o	±	+	+++	+++	o	+++	++++	+++	+	+	+
Connective tissue fibrinoid‡	Red	Red	o	o	o	o	o	+++	+++	o	+++	++++	+++	±	o	±
Vascular fibrinoid	Red	Red	++++	++++ Blue	++++	+++	++	o	o	+++	±	±	+++	+	++	+++
Vascular hyalin	Red	Red	++++	++++ Blue	++++	+++	++	o	o	+++	±	±	+++	±	++	+++
Connective tissue hyalin	Blue	Green	o	o	o	o	o	o	o	o	o	±	±	o	±	o

* The positive reactions are graded ± to ++++. Reactions of ++ to ++++ were considered significant.

† All of the material stained eosinophilic with hematoxylin and eosin and all except the hyalin of the meningioma yielded negative results with the following procedures: hemoglobin, iron, vitamin C, bile, glycogen, mucin, non-specific esterases, the Feulgen reaction. The connective tissue hyalin of the meningioma gave a positive reaction for esterases.

‡ The Schultz reaction, Congo red stain, and reaction for free carbonyl groups were ± instead of o for the fibrinoid in the gastric ulcer.

For comparison, simultaneous studies on the fibrinous exudate in 4 cases of uremic pericarditis have been included.

The tinctorial and histochemical methods employed are given below. The hematoxylin and eosin stain, Mallory's aniline blue stain, Masson's trichrome stain, and van Gieson's stain were performed in the conventional fashion.[16,17] The following procedures were carried out in accordance with the methods outlined in the monograph by Pearse[18] or as given by specific reference: oil red O, Nile blue sulfate, Sudan black B, and Congo red on frozen section,[17] toluidine blue stain at pH 5,[19] Best's mucicarmine, the Schultz reaction, the xanthoproteic reaction,[20] the reaction for free carbonyl groups of Ashbel and Seligman,[21] the Millon reaction, the periodic acid-Schiff reaction on frozen and paraffin sections, the reaction for protein-bound sulfhydryl groups of Barrnett and Seligman,[22] Gomori's modification of MacCallum's method for the demonstration of free potassium,[19] the Feulgen reaction, the non-specific esterase reaction of Nachlas and Seligman,[23] the hemoglobin procedure by the method of Dunn, bile pigment as depicted by the Fordine reaction after Glick,[24] vitamin C by the silver nitrate precipitation reaction, indole by the Ehrlich-Lison reaction, and calcium by the alizarin red method.

Results

The results with the procedures employed are given in Tables I and II. It may be seen by inspection of Table I that vascular fibrinoid and vascular hyalin gave similar tinctorial and histochemical patterns.

Table II

Similarly and Differently Reacting Tissues According to the Procedures Used in This Experiment

Similar reactions
1. Vascular fibrinoid and vascular hyalin
2. Connective tissue fibrinoid and fibrin
Different reactions
1. Vascular hyalin and connective tissue hyalin
2. Vascular fibrinoid and connective tissue fibrinoid
3. Connective tissue fibrinoid and connective tissue hyalin

Connective tissue fibrinoid from the sources utilized and the fibrin of uremic pericarditis gave similar histochemical and tinctorial patterns. The connective tissue fibrinoid of the rheumatoid nodule refers only

to the material in the center of the nodule and not to the partially necrotic palisade of cells at the periphery.

Vascular fibrinoid was encountered within the lumina and the walls of small arterioles, and in some instances as glomerular "emboli."[25] Vascular hyalin was found within and replacing the walls of small arteries and arterioles.[26] It was encountered also as glomerular nodules of the acellular type.[25]

Discussion

The listing of the positive and negative reactions in Table I emphasizes similarities and differences between vascular fibrinoid and vascular hyalin, connective tissue fibrinoid from a limited number of sources and connective tissue hyalin, and fibrin as deposited on the pericardial surfaces. These similarities and differences are summarized in Table II.

The similarities between vascular fibrinoid and vascular hyalin not only confirm previous observations[6,14,25,26] but by the inclusion of additional procedures in the present study lend further support to the view that these two substances are related pathogenetically. Vascular fibrinoid and vascular hyalin possess substances which yield the reaction of fats, lipids, aldehyde groups, free carbonyl groups, sulfhydryl groups, and free potassium. With the common stains the tinctorial properties of these two substances differ from those of collagenous fibers. Moreover, these two substances were non-reactive with procedures for calcium, indole, tyrosine, phenylalanine, tryptophan, and peptides. A series of additional procedures, listed with Table I, which gave negative results with the connective tissue lesions and fibrin, also gave negative results with the vascular lesions.

Connective tissue fibrinoid from the centers of rheumatoid nodules and the base of a peptic ulcer yielded the same reactions as fibrin deposited on the pericardium. These reactions differed in a major way from those of vascular fibrinoid and vascular hyalin. Connective tissue fibrinoid and fibrin emphasized those reactions which yield positive results for aldehyde groups, indole, calcium, tyrosine, phenylalanine, tryptophan, and peptides. In addition, fibrin gave slightly positive results for sulfhydryl groups and potassium and with Congo red. Particularly noteworthy were the negative reactions by these two substances for fats and lipids. With the common stains, connective tissue fibrinoid, fibrin, and vascular fibrinoid were of similar appearance.

Connective tissue hyalin was virtually non-reactive with the pro-

cedures used, with the exception of a positive reaction for non-specific esterases[23] yielded by the hyalin of the meningioma.

Previously we have discussed similarities in the location, tinctorial characteristics, histochemical reactions, and the pattern of microspectrophotographic absorption of vascular fibrinoid, vascular hyalin, and normal arteriolar smooth muscle.[6,14,25-27] These and other observations[28-30] have led to the concept that both vascular fibrinoid and vascular hyalin of arteriolar sclerosis are derived mainly from vascular smooth muscle. The present study lends additional inferential support to this view.

The view that vascular hyalin is derived mainly from vascular smooth muscle is not meant to imply that the admixture of connective tissue and connective hyalin with vascular hyalin in certain phases of arteriolar sclerosis does not occur. Indeed, this admixture has been described.[28,29] In arteriolar sclerosis, connective tissue hyalin is more likely to be encountered in the intimal and adventitial areas. With certain forms of experimentally induced arteriolar sclerosis[28,29] the connective tissue hyalin from these two areas may encroach upon and even replace the hyalin of the medial zone. Thus, various mixtures of vascular and connective tissue hyalin may develop in arteriolar sclerosis.

It would appear that a classification of fibrinoid into vascular and connective tissue types is indicated, based on the distinguishing characteristics of these substances. According to available information,[1] there is a possibility of different types of connective tissue fibrinoid. One type reveals characteristics similar to fibrin. This conclusion is in keeping with the observations of Craig and Gitlin[31] who utilized a different approach.

Summary

Vascular fibrinoid, vascular hyalin, connective tissue fibrinoid, connective tissue hyalin, and fibrin have been subjected to a series of tinctorial and histochemical procedures.

Vascular fibrinoid and vascular hyalin are tinctorially and histochemically similar.

Connective tissue fibrinoid from limited sources and fibrin as deposited on the pericardium are tinctorially and histochemically similar. These two substances differ from vascular fibrinoid and vascular hyalin.

Connective tissue hyalin differs from vascular fibrinoid, vascular hyalin, connective tissue fibrinoid, and fibrin and is virtually inert to several histochemical procedures.

According to the present and previous studies, vascular fibrinoid and vascular hyalin appear to represent alterations of arteriolar smooth muscle.

Connective tissue fibrinoid, from the lesions studied, appears to be similar in composition to fibrin.

It appears that vascular fibrinoid and connective tissue fibrinoid from certain sources should be considered as fundamentally different.

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A STUDY OF THREE STRAINS OF VACCINIA VIRUS IN STABLE CELL STRAINS L, LLC-M_1, AND HeLa*

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The suitability of stable strains of mammalian cells maintained in continuous culture for fundamental studies concerning host-virus relationships has been recognized by many workers. Investigative efforts with vaccinia virus, however, have been rather limited in this field. Scherer[1] noted that suspensions of vaccinia virus caused agglutination of mouse fibroblastic cells (strain L, Earle), but viral multiplication did not occur. Scherer and Syverton[2] propagated vaccinia virus through 15 passages in human epithelial cells (strain HeLa, Gey) and showed that the virus formed intracytoplasmic inclusion bodies and produced degenerative cellular changes; their findings were confirmed by Tyrrell.[3] Noyes and Watson[4] studied vaccinia virus in HeLa cells by means of the fluorescent antibody technique and found increasing amounts of particulate antigen in the cytoplasm of infected cells at 9, 16, and 24 hours after inoculation.

The present study was undertaken in order to compare the relative capacities of three stable cell strains to support growth of different strains of vaccinia virus and to investigate the cytologic effects of the viruses during infection. Three vaccinia viruses having varied backgrounds were selected which would be expected to provide a range of infectious properties—a neurotropic strain, a dermotropic strain, and a tissue culture strain. These viruses in conjunction with three strains of cells—two strains of mouse fibroblastic cells and a strain of human epithelial cells—presented nine possible combinations having interesting potentialities.

MATERIALS AND METHODS

Viruses

Mouse Neurotropic (WR) Strain. The virus was obtained as lyophilized mouse brain from the American Type Culture Collection and was maintained in this laboratory by intracerebral transfer in young

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mice (Swiss-Webster strain). Infected mouse brains were frozen, triturated, diluted with horse serum-saline* to form a 10 per cent suspension by weight, and stored at –45° C. For use, thawed preparations were centrifuged at 3,000 r.p.m. for 10 minutes and the supernatant fluid was employed as a 1:10 dilution of the virus. The LD_{50} titer for mice inoculated intracerebrally was $10^{-5.5}$ per 0.03 ml., and the infectious titer for rabbits inoculated intradermally was $10^{5.0}$ per 0.1 ml.

Rivers Strain. The virus was received as infected chick chorioallantois from Dr. W. H. Gaylord, Yale University School of Medicine, New Haven, Conn. Originally a dermotropic strain, it was established in tissue culture in 1931 by Rivers.[5] The virus was maintained in this laboratory by passage in the chorioallantoic membrane of 10-day-old chick embryos. Infected membranes were harvested within 48 to 72 hours after inoculation, and suspensions were prepared and stored as described above. The infectious titer for rabbits inoculated intradermally was $10^{5.0}$ per 0.1 ml.

Minnesota Strain. This virus has been described previously[1,2] and was supplied by Dr. W. F. Scherer, University of Minnesota School of Medicine, Minneapolis, Minn. Two sealed ampules were received, labeled "Vacc HeLa—$R_1$207, 4/5/55," which contained cultural fluid from the first passage of the virus in HeLa cells.[2] Serial passages in the chorioallantois of chick embryos were begun, and infected membranes were treated and stored as noted above. The infectious titer for rabbits inoculated intradermally was $10^{-5.0}$ per 0.1 ml.

Strains of Cells

HeLa strain (Gey), derived from a human cervical carcinoma,[6] was supplied by Dr. W. F. Scherer, University of Minnesota School of Medicine, Minneapolis, Minn.

L strain (Earle), derived originally from subcutaneous tissue of a C_3H strain mouse[7] and later established as a clone from a single isolated cell,[8] was supplied by Dr. W. R. Earle, National Institutes of Health, Bethesda, Md.

LLC-M_1 strain (Hull), derived from lymphoid tissue of a white mouse of unknown strain,[9] was supplied by Dr. R. N. Hull, Eli Lilly Laboratories, Indianapolis, Ind.

* Normal horse serum diluted 10-fold in physiologic saline solution and containing 500 units of penicillin per ml.

Cultivation of Tissue Cells

The procedures which were employed for cultivation of stock cellular cultures and for preparation of cultures in test tubes for viral studies were essentially similar to the techniques which have been described for HeLa cultures.[10,11] The nutritive fluid for HeLa cultures consisted of 75 per cent human ascitic fluid and 25 per cent Earle's balanced salt solution (BSS); 2 per cent chick embryonic extract was included in the medium when subcultures were prepared. The nutritive fluid for strain L and LLC-M_1 cultures consisted of 40 per cent Earle's BSS, 40 per cent normal horse serum, and 20 per cent chick embryonic extract. The nutritive fluids contained 500 units of penicillin and 100 μg. of streptomycin per ml. For histologic studies, cells were cultivated on 11 by 22 mm. coverglasses contained in Leighton tubes.*

Cultivation of Viruses

Nutritive fluids were removed and the cultures were washed with three changes of Hanks's BSS prior to inoculation. The initial inocula consisted of stock virus suspensions prepared as described above. One-tenth of 1 ml. of suspension was added to each culture, and after 30 minutes 0.9 ml. of maintenance medium was added to each tube. The maintenance medium consisted of 10 per cent normal horse serum in Earle-Hanks's (2:1) BSS and contained 100 units of penicillin and 50 μg. of streptomycin per ml. The cultures were incubated at 36° C. and were harvested within 48 to 72 hours after inoculation. Harvested cultures were stored at −45° C. in screw-capped tubes. For use, thawed preparations were centrifuged at 2,000 r.p.m. for 5 minutes and the cultural fluids were employed as inocula for fresh cultures. Uninoculated control cultures were included with each passage.

Assays of Viruses

Viral infectivity was quantitated in rabbits, HeLa cultures, and mice (WR strain of vaccinia virus). Serial 10-fold dilutions of cultural fluids were prepared in Hanks's BSS. One-tenth of 1 ml. of each dilution was inoculated intradermally into young adult rabbits (New Zealand strain) and into duplicate tubes of washed HeLa cultures, and 0.9 ml. of maintenance medium was added to each culture after 30 minutes. Final readings were made on the fifth day after inoculation. The infectious titer was termed the greatest dilution which

* Obtained from Microbiological Associates, Bethesda, Md.

(a) produced a dermal lesion in the rabbit; (b) produced cytopathogenic changes in HeLa cultures. Uninoculated control cultures were included with each titration *in vitro*. Five young mice (Swiss-Webster strain, average weight 12 to 15 gm.) were inoculated intracerebrally with 0.03 ml. of each dilution of the WR strain of vaccinia virus. Mice that died within 48 hours were disregarded in the final calculations. The 50 per cent end point of titration was determined according to the method of Reed and Muench.[12]

Immunologic Identification of Viruses

Neutralization tests were performed in rabbits and in HeLa cultures. Specific immune sera were obtained from the rabbits which were used for assay of the stock viral suspensions. The constant virus-varying serum technique was employed.[13] The sera were heated for 30 minutes at 56° C. prior to use, and serial two-fold dilutions were prepared in Hanks's BSS. An equal volume of the homologous virus preparation (containing approximately 100 infectious units per 0.1 ml.) was added to each increment of antiserum, and the serum-virus mixtures were left 1 hour at room temperature. One-tenth of 1 ml. of each mixture was transferred to duplicate HeLa cultures, 0.9 ml. of maintenance medium was added to each tube, and the cultures were incubated at 36° C. Final readings were made on the fourth day after inoculation.[14] Rabbits were inoculated intradermally with 0.1 ml. of each serum-virus mixture, and final readings were made on the fifth day after inoculation.[15] The neutralizing titer of an immune serum was termed the greatest dilution which (a) inhibited the formation of a dermal reaction in the rabbit; (b) inhibited the development of cytopathogenic changes in HeLa cultures.

Histologic Techniques

Coverglass cultures of tissue cells were washed, fixed, and stained with hematoxylin and eosin.[16]

Experimental Results

The inoculated cultures were examined daily for evidence of cellular changes which would indicate viral proliferation. Cytopathogenic changes which were observed readily in cultures examined *in situ* consisted of rounding, clumping, granulation, and shedding of cells.

Each passage of the three viruses in HeLa cultures and of the WR virus in L and LLC-M_1 cultures was accompanied by progressive degenerative changes, and tests for infectivity produced positive results. The initial passages of the Rivers and Minnesota strains in L

and LLC-M_1 cultures were accompanied by rounding and clumping of the cells, but no changes were observed during succeeding passages, and tests for infectivity after several passages produced negative results. Another series of passages of the latter viruses in L and LLC-M_1 cultures was begun, the results of which were as follows: (a) both viruses again failed to proliferate in L cultures; (b) the Rivers strain in LLC-M_1 cultures produced no cellular changes beyond the initial passage until the seventh transfer, when cellular clumping was observed; increasing degrees of cellular rounding, clumping, and granulation occurred during the eighth through the tenth passages, and from the eleventh passage onward marked to complete degeneration of the cultures occurred.

Investigative efforts were concentrated thereafter on the six cell-virus systems in which viral proliferation was manifested by cytologic changes and by tests for infectivity, i.e., the three viruses in HeLa cultures, the WR strain in L and LLC-M_1 cultures, and the Rivers strain in LLC-M_1 cultures.

Viral Assays

A composite of the results of the quantitative tests for infectivity in rabbits, HeLa cultures, and mice is presented in Table I.

The experimental data showed that HeLa cultures provide a more sensitive and accurate system than rabbits and mice for the assay of vaccinia viruses. Huang[17] pointed out that the use of tissue cultures

TABLE I

Accumulative Data Concerning the Vaccinia Viruses Cultivated in Cell Strains L, LLC-M_1, and HeLa

Virus,* passage number, cell strain	Number of days in culture	Cumulative log of dilution of original viral inoculum	Negative log of infectious titer		
			Rabbits	HeLa cultures	Mice (LD_{50})
WR P24 L	228	25	4.0	5.0	5.0
WR P20 LLC-M_1	228	21	5.0	5.0	4.5
WR P22 HeLa	155	25	4.0	6.0	4.5
Ri P10 LLC-M_1	125	12	4.0	5.0	
Ri P20 HeLa	159	22	4.0	5.0	
Minn P20 HeLa	159	22	4.0	5.0	

*Example: WR strain, passage 24, L cells.

for viral quantitation eliminates ". . . . the variable of individual animal reactivity." In addition, titrations in HeLa cultures were easier and simpler to perform than in animals, and the experimental conditions were more readily controlled.

Neutralization Tests

The propagated viruses were inhibited specifically by immune sera prepared against the original viruses. In general, the viruses were suppressed in all dilutions of the antisera as compared with control inocula, and relatively mild dermal reactions and cytopathogenic changes were produced even in the presence of low concentrations of antisera. No attempt was made to obtain highly potent antisera for the tests, since the primary object of the tests was identification of the cultured viruses. The neutralizing titers of the immune sera are shown in Table II.

TABLE II

Neutralizing Titers of the Specific Immune Sera in Rabbits and HeLa Cultures

Virus, passage number, cell strain	Neutralizing titer	
	Rabbits	HeLa cultures
WR P25 L	1:64	1:512
WR P21 LLC-M_1	1:64	1:64
WR P21 HeLa	1:512	1:512
Ri P11 LLC-M_1	1:64	1:256
Ri P21 HeLa	1:8	1:256
Minn P21 HeLa	1:256	1:512

The tissue culture method was found to be more sensitive and the results were more readily reproducible *in vitro* than *in vivo*. Similar findings have been reported by Ledinko *et al.*[14] with poliomyelitis virus in tissue cultures and mice.

Morphologic Changes in Infected Cells

The progressive degenerative changes induced by replication of the vaccinia viruses followed the same pattern in the three cellular strains, i.e., cytoplasmic retraction, cellular aggregation, increasing granularity, fragmentation, and eventual death. Areas of focal degeneration were more obvious in HeLa cultures than in L and LLC-M_1 cultures, doubtless because HeLa cells grow in syncytial sheets while the mouse fibroblastic cells remain relatively discrete during multiplication.

The WR strain of vaccinia virus in L cultures produced cellular rounding and clumping within 24 hours. Moderate to marked cytopathogenic changes consisting of cytoplasmic granulation and fragmentation, and nuclear pyknosis and karyorrhexis were apparent within 48 hours after inoculation. Cytoplasmic extensions of infected cells frequently showed bulbous areas, the significance of which is unknown. At 72 hours most of the cells were detached from the surfaces of the glass. Numerous intracytoplasmic inclusion bodies were present at 24 hours. The inclusions varied in size and shape and generally were surrounded by a distinct halo; some inclusions were

perinuclear, and others were scattered throughout the cytoplasm. Figure 1 shows uninfected L cells, and the cytologic changes described above are depicted in Figures 2 to 6.

Similar cytopathologic changes were observed in LLC-M_1 cultures infected with the WR strain. The cytoplasm of infected LLC-M_1 cells, however, tended to become more granular and to fragment more readily than that of the L cells, and although inclusion bodies were numerous, they were not well demarcated from the surrounding cytoplasm. Figure 7 shows uninfected LLC-M_1 cells, and the effects of viral multiplication are presented in Figures 8 and 9. Beginning with the eleventh passage, the Rivers strain of virus produced more rapid and destructive changes in LLC-M_1 cells than did the WR strain. Marked cellular injury was evident within 24 hours after inoculation (Fig. 10), and at 48 hours only scattered cells remained adherent to the surfaces of the glass. Numerous well defined inclusion bodies were present at 24 hours, and relatively undamaged cells often contained several inclusions (Fig. 11).

All three strains of vaccinia virus produced practically indistinguishable changes in HeLa cultures. Focal areas of degeneration consisting of clear central portions bordered irregularly by deeply staining necrotic cells appeared within 24 hours. Subsequently, the plaques enlarged peripherally as neighboring cells became involved, and coalescence of adjacent plaques resulted in widespread damage within 48 hours after inoculation. Uninfected HeLa cells are shown in Figure 12. A typical plaque is depicted in Figure 13, and Figure 14 shows the appearance of infected cultures 48 hours after inoculation. Characteristic vaccinial inclusion bodies are shown in Figure 15. Similar cytologic changes in HeLa cultures infected with vaccinia virus have been described by Scherer and Syverton.[2]

Discussion

Viral propagation was demonstrated in six of the nine cell-virus combinations which were tested. A sufficient number of serial passages was conducted so that the infectious titers of the original inocula were exceeded widely, and material from the final passage of each series contained infective virus which was identical immunologically with the original inoculum. Infected cells underwent progressive degeneration and typical inclusion bodies were formed. Finally, adequate controls excluded the possibility of non-specific reactions and of microbial contamination. Cultivation of three strains of vaccinia virus in HeLa cultures confirms and extends the findings of Scherer and Syverton,[2]

and vaccinia virus has been propagated in cells of strains L and LLC-M_1, which heretofore has not been reported.

The ability of various strains of vaccinia virus to multiply in HeLa cells requires little comment, since this cell strain was derived from a human epidermoid carcinoma and this virus is epitheliotropic. Moreover, HeLa cells have been shown to support the growth of a number of animal viruses.[3,11,18-22] With respect to the mouse fibroblastic cell strains, the infectious capacities of the vaccinial strains may be related to the known backgrounds of these viruses. Proliferation of the WR strain in both L and LLC-M_1 cells may be attributed to adaptation of the virus to murine tissues as the result of repeated passage in the mouse. Although vaccinia virus is predominately dermotropic, the production of neurovaccinia from a dermotropic strain involves a change in the pathogenic properties of the virus. Neurotropic vaccinia virus retains its ability to proliferate in the skin, and acquires the capacity to infect other tissues, particularly those of mesodermal origin.[23-25] The ease with which the WR strain was cultivated in L and LLC-M_1 cultures is indicative of the increased affinity of neurovaccinia for cells of mesodermal origin.

The Rivers strain of vaccinia virus, which proliferated in LLC-M_1 cultures on the second trial, has been cultivated *in vitro* for many years and has been employed for investigative purposes up to the present time. Understandably, this virus should possess the ability to multiply under varied conditions. While retaining its dermotropic properties, the virus has had ample opportunity to adapt to cells other than those of ectodermal origin. On the other hand, the Minnesota strain behaves as a virus with predominately dermotropic properties. In this laboratory, it proliferated abundantly in rabbit dermis whether inoculated by scarification or intradermally, whereas the WR and Rivers strains, inoculated intradermally, produced consistently higher titers than by scarification. The Minnesota strain was maintained in Scherer's laboratory in the chorioallantois of chick embryos and was cultivated in HeLa cells,[2] but it did not multiply in L cells.[1] During the present study, the virus was cultivated readily in HeLa cells, but failed to proliferate in mouse fibroblastic cells. The effect of prolonged cultivation in HeLa cells on the ability of the Minnesota strain to propagate in L and LLC-M_1 cells is being investigated.

Scherer[1] reported that the Minnesota strain caused "agglutination" of L cells without viral multiplication. The same phenomenon was observed with the Minnesota and Rivers strains in cultures of L and LLC-M_1 cells during the present study. The appearance of the cells

in situ suggested that virus in the original inocula initiated infection; if viral reproduction then occurred, perhaps "incomplete" virus was formed which lacked the power to infect fresh cultures. That incomplete but not necessarily non-infective virus also may have been formed is indicated by the results of the second attempt to cultivate the Rivers strain in LLC-M_1 cells, when cellular aggregation occurred during the first passage but no further changes were noted until the eighth transfer. Infective virus was present during the fourth, tenth and later passages, and at present the virus has been subcultured 18 times. Two possibilities are suggested: (a) infective but non-cytopathogenic virus was formed during the early passages; (b) the virus remained latent for several passages followed by the formation of infective and cytopathogenic virus. The above observations allow speculation as to whether "incomplete" or "latent" vaccinia virus may be produced under certain cultural conditions. Further investigation is required to clarify the situation.

The rapid cellular degeneration resulting from multiplication of the vaccinia viruses in HeLa cultures resembles the marked action of other viral agents upon these cells, including the viruses of poliomyelitis, herpes simplex, and equine encephalomyelitis.[2,10,20] Strain L cells have been shown to support growth of the viruses of pseudorabies, herpes simplex, lymphocytic choriomeningitis, pseudolymphocytic choriomeningitis, and Western equine encephalomyelitis,[26-28] and herpes simplex virus has been cultivated in LLC-M_1 cells,[29] but the cytopathogenic effects of the viruses was not clearly stated. The present study apparently represents the first definitive investigation concerning the degenerative effects of a virus during growth in L and LLC-M_1 cells.

Summary

A study of the relative capacities of different strains of vaccinia virus to propagate in three stable strains of mammalian cells and of the cytologic changes induced by viral infection has been presented. Differences in the abilities of the viruses to proliferate in the cellular strains were observed. Viral reproduction was demonstrated in six of the nine cell-virus systems that were investigated. All three viral strains—WR, Rivers, Minnesota—multiplied in HeLa cultures; the first two multiplied in LLC-M_1 cultures, and only the WR strain multiplied in L cultures. Viral proliferation was characterized by the formation of typical intracytoplasmic inclusion bodies and by the appearance of progressive degenerative changes which produced complete destruction of the cultures within 48 to 72 hours following

inoculation. The viruses produced similar cellular changes in HeLa cultures, but the Rivers strain produced more rapid and destructive changes in LLC-M_1 cultures than did the WR strain. HeLa cultures were found to provide a more sensitive and accurate system than the rabbit for quantitation of vaccinial viruses and antibodies. The pathogenicity of the viruses for the experimental animals was reduced somewhat during cultivation *in vitro*. The possibility of the occurrence of immature forms of vaccinia virus during attempts to cultivate the Rivers and Minnesota strains in L and LLC-M_1 cells was suggested.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

All preparations were fixed in Zenker's-acetic acid solution and stained with hematoxylin and eosin.

FIG. 1. Uninfected strain L cells. × 598.

FIG. 2. WR strain of vaccinia virus in L cells, 24 hours after inoculation. There are bulbous areas along cytoplasmic extensions. × 598.

FIG. 3. WR strain of vaccinia virus in L cells, 48 hours after inoculation. Cellular aggregation is evident and most of the cells show signs of degeneration. × 126.

FIG. 4. WR strain of vaccinia virus in L cells, 72 hours after inoculation. Only scattered, degenerate cells remain on the glass. × 126.

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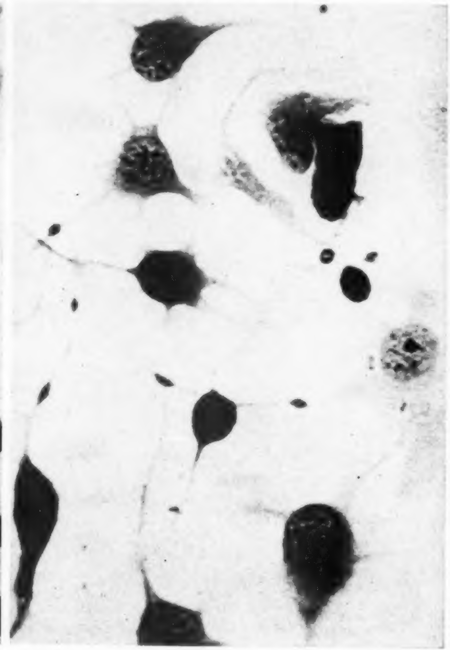

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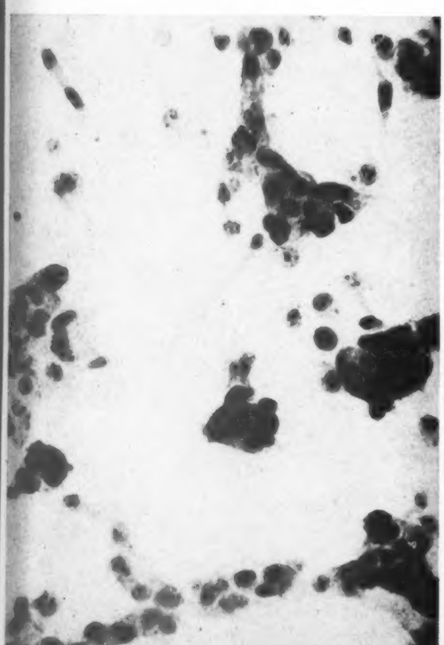

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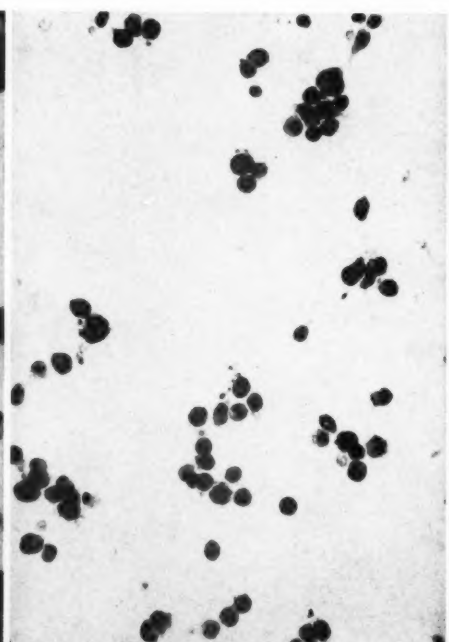

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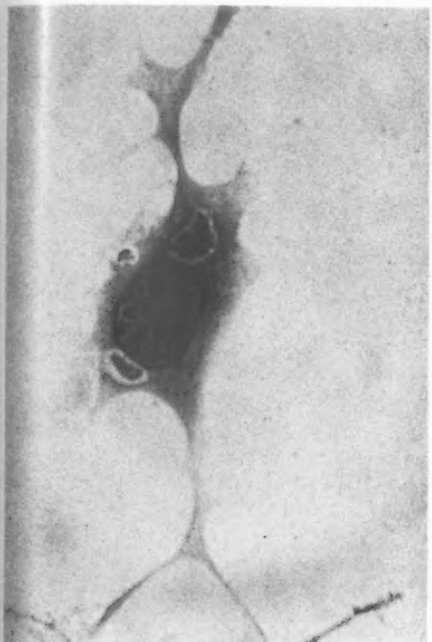
FIG. 5. WR strain of vaccinia virus in L cells, 24 hours after inoculation. The cell shown contains well defined intracytoplasmic inclusion bodies. × 1,134 (oil immersion).

FIG. 6. WR strain of vaccinia virus in L cells, 48 hours after inoculation. Of note are cytoplasmic granularity and fragmentation. × 1,134 (oil immersion).

FIG. 7. Uninfected LLC-M_1 cells. × 598.

FIG. 8. WR strain of vaccinia virus in LLC-M_1 cells, 24 hours after inoculation. Cellular rounding and clumping are evident. × 126.



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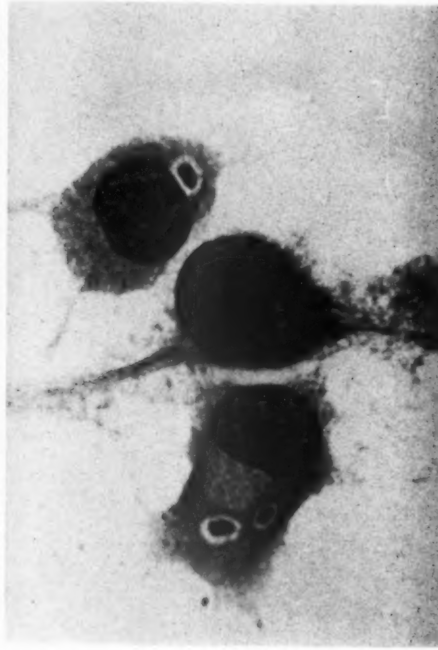

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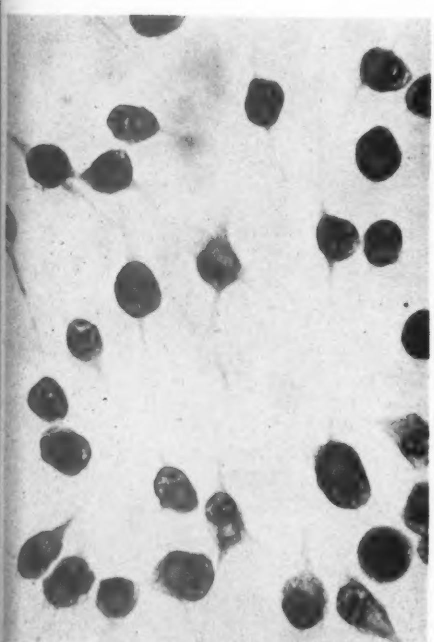

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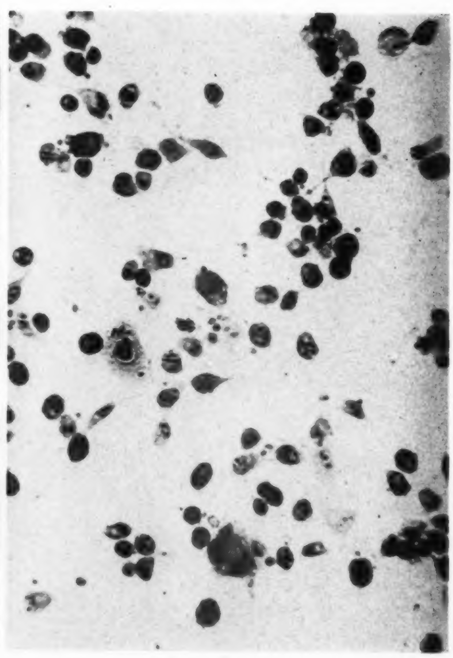

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FIG. 9. WR strain of vaccinia virus in LLC-M_1 cells, 24 hours after inoculation. The giant cell contains several homogeneous inclusion bodies. × 1,134 (oil immersion).

FIG. 10. Rivers strain of vaccinia virus in LLC-M_1 cells, 24 hours after inoculation. Comparison with uninfected LLC-M_1 cells (Fig. 7) reveals the marked and rapid degenerative effect of this virus. × 598.

FIG. 11. Rivers strain of vaccinia virus in LLC-M_1 cells, 24 hours after inoculation. The cell on the left contains several well demarcated inclusion bodies. × 1,134 (oil immersion).

FIG. 12. Uninfected strain of HeLa cells. × 598.

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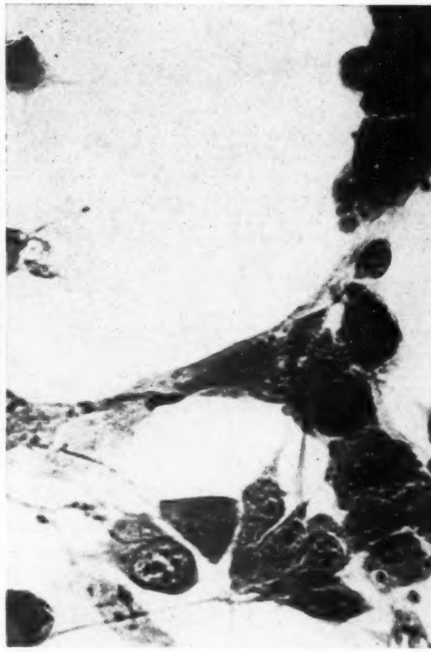

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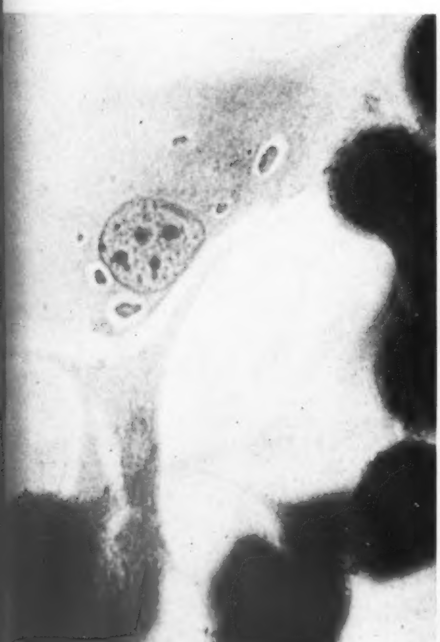

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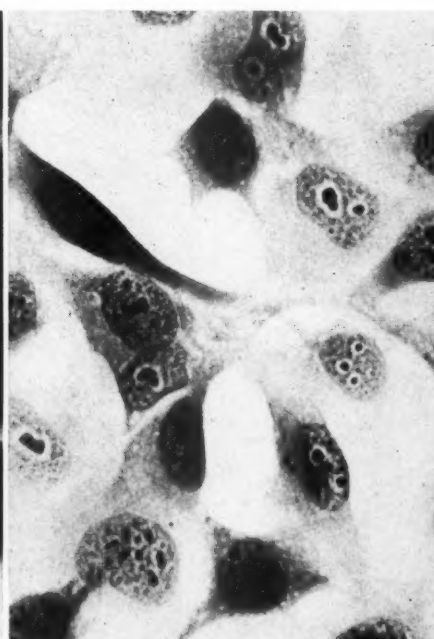

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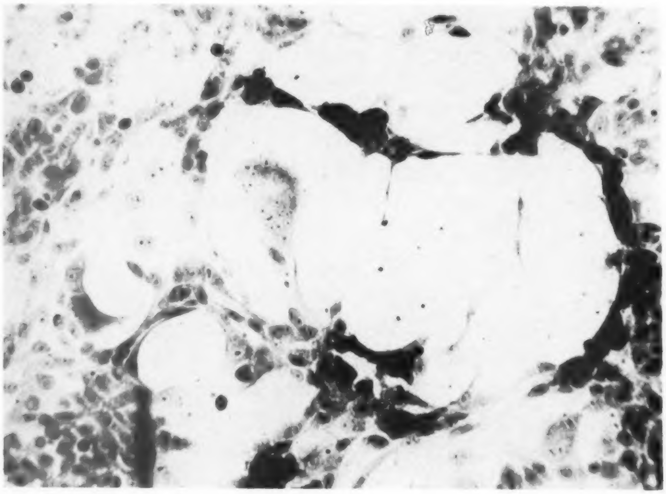
FIG. 13. WR strain of vaccinia virus in HeLa cells, 24 hours after inoculation. A characteristic early plaque is shown. × 126.

FIG. 14. Rivers strain of vaccinia virus in HeLa cells, 48 hours after inoculation. Only clumped and granular cells remain around the sites of the original plaques. × 75.

FIG. 15. Rivers strain of vaccinia virus in HeLa cells, 48 hours after inoculation. The nuclei have an irregular, shrunken appearance. One of the cells contains a distinct inclusion body. × 1,134 (oil immersion).



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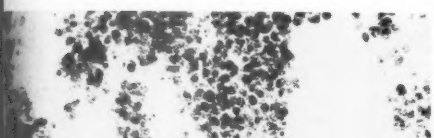

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NEPHROTIC GLOMERULONEPHRITIS*

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It has been apparent for many years that glomerulonephritis may present a pleomorphic clinical picture. On the basis of clinical and laboratory findings, the disease has been separated into two types: nephritic glomerulonephritis (Ellis type I) and nephrotic glomerulonephritis (Ellis type II).[1] Nephritic glomerulonephritis is characterized by a history of streptococcal infection (Lancefield group A, usually type 12),[2] a characteristic latent period, gross hematuria, hypertension, slight edema, oliguria, albuminuria, elevated antistreptolysin o titers, and a good prognosis (85 to 90 per cent recovery). Nephrotic glomerulonephritis, on the other hand, is delineated by an absence of any preceding history of bacterial infection, normal antistreptolysin titers, insidious onset of marked edema, albuminuria without significant hematuria, ascites, hyperlipemia, normal blood pressure, absence of azotemia, and a poor prognosis. From the differences enumerated above it is not surprising that many investigators believe that these types of glomerulonephritis are distinct diseases.[1] Clinically, it may be impossible to separate them from each other and from amyloidosis, diabetic glomerulosclerosis, chronic pyelonephritis, and nephrosclerosis.[3] However, these diseases generally are distinguishable pathologically.

Certain cases of nephrotic glomerulonephritis (nephrosis) in children in which hematuria, hypertension, and azotemia are absent have been called pure lipid nephrosis. Many of these children eventually develop hematuria, azotemia, or hypertension and are said by the clinicians to show a transition from a nephrotic to a nephritic picture. Inasmuch as these so-called nephritic patients will show pathologically only evidence of nephrotic glomerulonephritis, it appears that the different manifestations of these cases are all part of the natural evolution of nephrotic glomerulonephritis.

The nature of the microscopic changes in nephrotic glomerulonephritis have been controversial for years. When death occurs early in the course of this disease from intercurrent infection, the structural glomerular changes may be so minimal that some investigators have claimed that no glomerular lesion exists and that the tubular

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lesions indicate a primary tubular disease. While the lesions of the glomeruli may be so minimal that they are overlooked, a small number of definitely scarred glomeruli usually are present.

With progressive activity of the disease, the glomerular changes become more apparent and thus well defined glomerular lesions usually are seen in the older child and adult with nephrosis. Bell[4] described thickened basement membranes about the glomerular capillaries in the nephrosis of adults and noted a suggestion of vacuolization in these membranes. He designated this lesion as membranous glomerulonephritis. McManus[5] noted vacuolization in the mesangium of the glomerulus in nephrosis and demonstrated neutral fat in the vacuoles.

In addition to the membranous lesion of nephrotic glomerulonephritis, Ellis[1] described a peculiar nodular glomerular scar. It was characterized by an enlarged glomerulus with hyaline scar tissue in the center of each lobule of the tuft surrounded by thick-walled capillaries. This lesion may be mistaken for diabetic glomerulosclerosis. Bell[4] called this lesion chronic glomerulonephritis with the clinical picture of lipid nephrosis of the mixed type. Allen[6] uses the term, chronic lobular glomerulonephritis.

The finer structure of the glomeruli of nephrotic glomerulonephritis is not well known. The present study is an attempt to use newer techniques to elucidate the different morphologic forms of this disease.

Methods

The basic histologic procedure of this study was the use of the periodic acid silver methenamine sequence which reveals the fine structures of the glomerular connective tissue in health and disease better than any other technique. It stains essentially the same structures as the periodic acid-Schiff (PAS) stain but with far greater contrast. This permits the resolution of delicate structures which would be missed by the PAS stain. The use of a gold toning procedure seems to exaggerate the contrast even more. It must be stressed that thin sections, 1 to 3 μ thick, are a prerequisite for the visualization of fine details in the glomerulus.

Periodic Acid Silver Methenamine Technique

Formalin, Carnoy's, and Bouin's fixatives give satisfactory results, but fixation in Zenker's or osmic acid solutions is not adequate for this procedure. Due to the high intensity of the stain, sections should be no thicker than 1 to 3 μ.

Procedure

Hydrate sections to distilled water containing 0.5% periodic acid at room temperature for 15 minutes
Wash well in distilled water
Place in Gomori's silver methenamine solution at 45° to 50° C. for 1½ to 3 hours (Gomori's stock solution contained 100 cc. of 3% methenamine and 5 cc. of 5% silver nitrate); add 6 cc. of 5% borax per 50 cc. of stock solution

Wash in distilled water
Tone in 0.2% gold chloride solution for 2 minutes
Wash in distilled water
Place in 3% sodium thiosulfate for 2 minutes
Wash well in tap water
Counterstain with hematoxylin and eosin, or any other suitable stain

Sections may be removed from the silver bath at any time, rinsed in distilled water, and examined under the microscope. If further stain is needed, they may be rinsed in distilled water and replaced in the silver bath. If the slides are overstained, an extremely dilute solution of potassium ferricyanide may be used to destain them to the desired intensity.

Counterstaining with acid dyes is progressively inhibited by the silver stain in much the same way as with osmic acid. Nuclear staining by the silver is seen with prolonged exposure to the silver methenamine solution; hydrolysis of nucleic acids occurs and a silver Feulgen reaction results.

This study is based on the examination of the kidneys of 20 cases of nephrotic glomerulonephritis which were divided into four groups: 6 cases presented minimal glomerular lesions; 6, moderate glomerular lesions; 3, the lesion of chronic lobular glomerulonephritis; and 5, chronic membranous glomerulonephritis.

Results and Discussion

The normal glomerulus in this discussion is considered to be a tuft of capillaries invested by the glomerular epithelium and the prominent subepithelial basement membrane. This basement membrane is continuous with the basement membrane of Bowman's capsule and the proximal convoluted tubules. The capillaries of the tuft are covered by this basement membrane in the same manner as the serosa covers the small bowel.[7-14] A mesentery-like structure thus formed may be called the glomerular stalk or mesangium. Two types of cells are present within the subepithelial basement membrane. Morphologically, there is little difference between them but they may be distinguished by their location and by what they do. The endothelial cell lies flattened against the basement membrane and it forms the endothelial tube of the vessel. The mesangial connective tissue cell lies in the mesangial stalk and it is surrounded by silver-positive fibrillar tissue which is of an irregular spongy nature and stains like the material of the basement membrane. Since this fibrillar material has qualities which are different from other fibrillary connective tissue, the term fibromucin is considered appropriate.

With disease, the endothelium of the capillaries may be separated from the overlying basement membrane by edema, exudate, or other material. Very soon another basement membrane becomes visible adjacent to the endothelium; thus a pericapillary connective tissue space

bounded by basement membranes is formed. Although electron microscopists cannot clearly demonstrate an endothelial basement membrane,[14] the fact that it rapidly becomes visible in disease suggests that normally a thin basement membrane of at least molecular thickness must exist. Aterman[15] and Gersh and Catchpole[16] have shown that basement membranes may thicken rapidly in disease.

The changes in inflammation of connective tissue are predominately the result of exudation and reactions by the fibroblasts. In the glomerulus the same principle holds true. Changes in the glomerular connective tissue other than exudation would appear to be the result of metabolic changes in the connective tissue cells (mesangial cells) so that they lay down more basement membrane substance, fibromucin, or hyalin. The endothelial cells appear to be of little significance since they are prevented from budding by the intact basement membrane. What is the nature of the tissue in the glomerular scars? It is not collagen, reticulin, or elastin, as can be demonstrated by histologic and electron microscopic techniques. It is composed of two materials: hyalin, an eosinophilic material identical to that seen in sclerotic arterioles, and fibromucin, which stains the same as basement membranes. With the periodic acid silver methenamine stain, hyalin is silver-negative but fibromucin and basement membranes are silver-positive.

Nephrotic Glomerulonephritis in Children

In the nephrotic child who dies of intercurrent infection relatively early in the course of the disease, changes in the structure of the glomeruli are minimal (Fig. 1). At the most, a slight increase in the number of mesangial connective tissue cells may be seen (Fig. 2). The tubules, on the other hand, may show striking changes with the accumulation of hyaline droplets and fatty vacuoles in the proximal convoluted segments.

The significance of the above cases is not that there is little glomerular change in nephrotic glomerulonephritis but that many of these children die before well defined lesions have developed. In those instances in which certain authors claim that there are no glomerular lesions in lipid nephrosis, we feel sure that their cases were either milder or earlier stages of the disease.

Some children die with azotemia complicating their nephrotic picture following persistent active disease. In these patients we see definite evidence of the destructive nature of the process on the glomeruli. An increase in connective tissue in the center of each

glomerular lobule and some variable thickening of pericapillary basement membranes may be seen (Figs. 3, 4, and 5). The lumina of some of the capillaries may be partially blocked by lipid-filled cells (Fig. 6). At this stage tubular atrophy and dilatation is very evident and often appears to be out of proportion to the degree of glomerular change.

Nephrotic Glomerulonephritis in Older Children and Adults

Older children and adults do not succumb as frequently to fatal intercurrent infections and thus tend to die of their disease. The increased maturity of their defense mechanisms may be the answer for this. The more advanced glomerular lesions are of three general types: the moderate scar of late nephrosis of childhood, the exaggerated form of this lesion in adults (chronic lobular glomerulonephritis), and the chronic membranous lesion.

The kidney of chronic lobular and chronic membranous glomerulonephritis may either be small or near normal size depending on the degree of tubular atrophy. The kidney with moderately scarred glomeruli usually is near normal size since tubular atrophy generally is slight in this lesion. Fatty and hyaline droplets may be found in the tubules. Interstitial fibrosis and arteriosclerosis tend to be proportional to the degree of tubular atrophy. In chronic nephritic glomerulonephritis the old glomerular scars have been removed, so that only a fraction of the original number of glomeruli remain. With the periodic acid silver methenamine stain, various stages of resorption of the glomerular scars of chronic nephritic glomerulonephritis may be demonstrated. Even in the shrunken fibrotic kidneys of chronic nephrotic glomerulonephritis an approximately normal number of scarred glomeruli may be seen.

Chronic lobular glomerulonephritis is characterized by large glomeruli, each lobule of which is distended by a central mass of cellular scar tissue (Fig. 7). The individual lobules tend to be club-shaped and a few residual thick-walled capillaries lie at the edge of the lobules. The central connective tissue scar (fibromucin and hyalin) is laid down in the region of the mesangium and as it enlarges it presses the capillaries to the periphery (Fig. 8). The earlier stages of this process may be seen in cases showing the moderate glomerular scars in which the scarring of each lobule has just started (Fig. 4). In some of the far advanced cases of chronic lobular glomerulonephritis silver-negative hyalin is deposited between the fibers of the connective tissue

(fibromucin) of the central scars and between the two layers of basement membrane about the capillaries. This may become so extreme that little silver-positive basement membrane substance remains in the glomerulus. In far advanced lesions few patent capillaries remain in the glomeruli. Early in the evolution of this lesion, the moderate scar, double contoured, thin basement membranes surround the capillaries; i.e., an epithelial basement membrane and a thickened endothelial basement membrane separated by connective tissue fluid are visible. Later more glomerular connective tissue is laid down (fibromucin) so that the capillaries are surrounded by dense scar. Very late in the evolution of this lesion the capillaries are obliterated and only nodular masses of scar tissue remain.

To the beginner, the lesion of chronic lobular glomerulonephritis looks like that of diabetic glomerulosclerosis. The absence of extensive hyaline arteriolar sclerosis of the afferent arteriole, the well developed cellularity of the lobular scars, the uniform involvement of all glomeruli, and the evidence of marked capillary obliteration clearly distinguish this lesion from that seen in diabetes mellitus.

The membranous form of nephrotic glomerulonephritis shows a characteristic red hyaline thickening of the basement membranes about the capillaries of the glomeruli with the hematoxylin and phloxine stain (Fig. 9). The glomeruli show some increased cellularity and often the capillary lumina are narrowed by the thickened walls. In rare glomeruli an epithelial crescent may be present. The glomerular basement membrane is thickened from two to four times. In describing this change, Bell[4] noted a vacuolated appearance of the membranes. In the present investigation it has become apparent that this lesion is the result of a peculiar modification of the epithelial basement membrane of the glomerulus. With the periodic acid silver methenamine stain this thickened membrane resolves itself into a much less thick epithelial membrane from which protrudes externally innumerable short silver-positive projections of club or mushroom shape. Between these projections is a silver-negative hyaline material which is in a droplet form (Fig. 10). This hyalin may be differentiated from overlying epithelial cytoplasm by Masson's trichrome stain in which the hyalin stains green and the cytoplasm rose. The PAS stain shows the same structure but there is insufficient contrast to make the lesion clear. In certain early lesions it appears that the hyaline droplets accumulate within the epithelial basement membrane. As they enlarge they push peripherally. When they are sufficiently large the external layer of basement membrane is no longer visible and the hyaline drop-

lets appear to be separated from the structure by silver-positive clubs. This hyalinosis of the basement membrane may become so extreme that little silver-positive material remains (Fig. 11). This alteration of the basement membrane is seen both in the chronic lobular and the chronic membranous lesions. The significance of the hyalin is conjectural. It appears likely that hyalin is an abnormal connective tissue deposit resulting from chronic injury to the cells of the mesangium which are responsible for maintaining the connective tissue spaces and basement membranes of the glomerulus.

In summary, the lesions of nephrotic glomerulonephritis may be divided into the minimal lesion of childhood in which death is usually due to intercurrent disease, the moderate lesion of children and young adults who die of renal failure, the chronic lobular lesions, and the membranous lesions of adults. An explanation as to why some adults develop the chronic lobular lesion and others the membranous lesion is not available.

The Biologic Relationship of Acute and Chronic Glomerulonephritis (Nephritic Glomerulonephritis, Ellis Type I) and Nephrotic Glomerulonephritis (Ellis Type II)

A large number of differences, epidemiologic, clinical, and morphologic, exist between nephritic and nephrotic glomerulonephritis. In spite of these differences there is evidence to suggest that they belong to one family.

It has been shown that both nephritic and nephrotic patients possess circulating antiglomerular auto-antibodies. The antibody titers rise with activity and fall following remission,[17] and the reaction is complement binding.[18] This fact correlates with the known depression of blood complement in the acute phases of the diseases.[19] It is further substantiated by the demonstration that human gamma globulin is bound to glomeruli in both nephrotic and nephritic glomeruli, the gamma globulin being an antiglomerular auto-antibody.[20]

With acute diffuse glomerulonephritis it appears that a streptococcal (Lancefield group A, type 12) infection usually triggers the auto-antibody production. It is postulated that a streptococcal product combines with the glomerular connective tissue to form an antigen which results in antibody formation. Even though glomerular connective tissue is not in itself antigenic, it combines like a haptene with the antibody and injury to the glomerulus of the Arthus type results. After a variable time the antibody falls to unreacting levels and the

injurious action ceases. Healing or scarring depends mainly on the severity and the duration of the injury. In most cases resolution is almost complete.

In nephrotic glomerulonephritis no triggering mechanism has been definitely established. The process is often gradual in onset and shows remissions and exacerbations. An auto-antibody formation similar to that of acute glomerulonephritis appears to be present but its mechanism is still unknown.

The Natural History of Nephrotic Glomerulonephritis

As a result of the high rate of death of nephrotic children from bacterial infection, the long-term effect of the disease on renal function is difficult to evaluate. In the edematous phase, hypoproteinemia, hypogammaglobulinemia, and other non-specific factors result in a high incidence of pneumococcal or streptococcal infections. Infection plus a few cases of gross electrolyte disturbance or heart failure result in about a 50 per cent rate of death in this phase of the disease. With the advent of antibiotics this incidence of death from infection will decrease so that a larger proportion of nephrotic children will live out the disease process.

Rennie,[21] in a group of 29 cases of nephrotic glomerulonephritis, found only 3 in which apparently complete healing had occurred after 7 to 15 years. Davson and Platt[3] reported 8 of 34 patients with this disease alive and well but 3 of the 8 patients showed albuminuria. Schwarz and associates[22] found only 4 of 40 nephrotic patients completely healed long after the onset of their disease. These figures indicate that in many patients progressive glomerular injury results in uremia. It is unfortunate that the figures of survival are not expressed in 5, 10, and 20-year periods as for cancer. It is impossible at the present time to estimate the relative prognosis of a patient in various phases of this disease.

With the advent of hormone, antibiotic, and other forms of therapy a larger proportion of patients survive the edematous phase of the disease but albuminuria often persists. We can hope that with this therapy a smaller percentage will develop azotemia or hypertension.

Summary

On clinical, pathologic, and natural historical grounds, nephrotic glomerulonephritis and nephritic glomerulonephritis have similarities and differences. It appears that a peculiar auto-antibody injury is

present in both processes but the trigger mechanism has not been discovered for nephrotic glomerulonephritis.

Pathologically, the types of glomerular lesions seen in nephrotic glomerulonephritis have been divided into the minimal lesion of childhood, the moderate lesion of the uremic child or young adult, the chronic lobular lesion, and the membranous lesion. Certain new observations of these lesions have been discussed.

It is hoped that future presentations of clinical material on nephrotic glomerulonephritis will make use of 5, 10, and 20-year survival statistics so that a better concept of ultimate prognosis may be reached.

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LEGENDS FOR FIGURES

FIG. 1. Nephrotic glomerulonephritis; minimal lesion in a child with lipid nephrosis. Hematoxylin and phloxine stain. × 385.

FIG. 2. Nephrotic glomerulonephritis; minimal lesion in a child with lipid nephrosis. The prominent dark-staining mesangial material filled with nuclei is the outstanding glomerular lesion. Silver methenamine, hematoxylin and eosin stains. × 1,000.

FIG. 3. Nephrotic glomerulonephritis; moderate lesion. There is an accumulation of fibromucin in the center of each lobule. Hematoxylin and phloxine stain. × 345.

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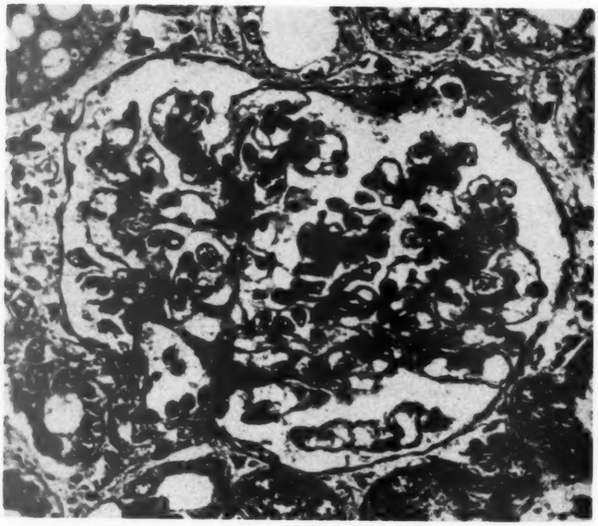

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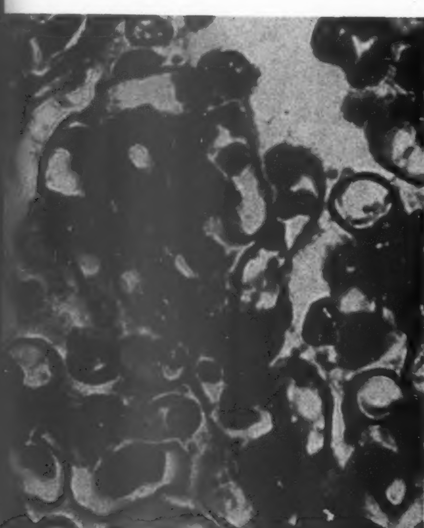

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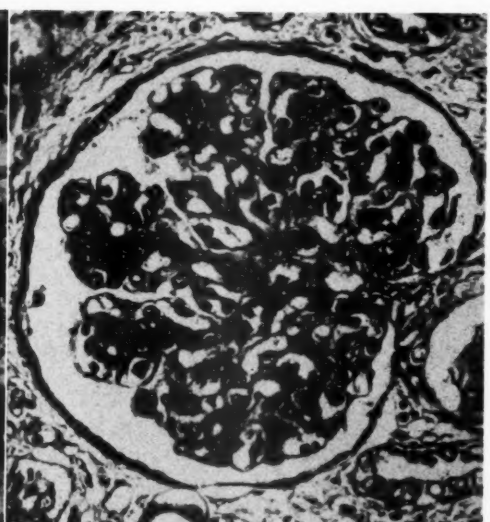

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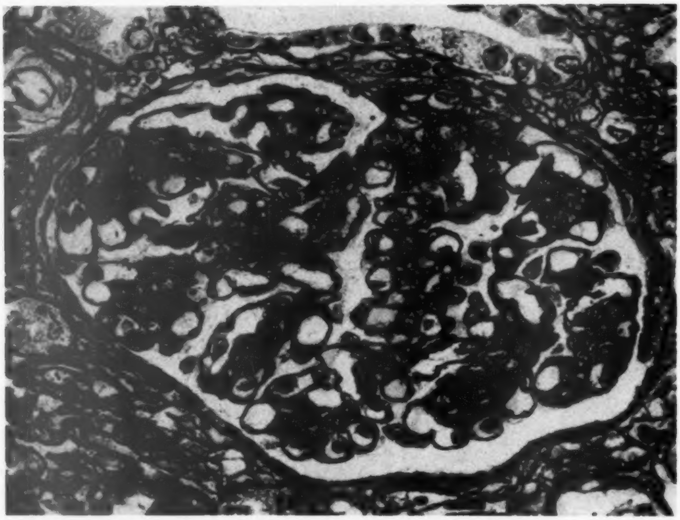
FIG. 4. Nephrotic glomerulonephritis; moderate lesion. This is similar to Figure 3 but is stained with silver methenamine, hematoxylin and eosin. × 490.

FIG. 5. Nephrotic glomerulonephritis; moderate lesion. The glomerulus shows evidence of reactive thickening of the basement membrane. Silver methenamine, hematoxylin and eosin stains. × 450.

FIG. 6. Nephrotic glomerulonephritis; moderate lesion. This glomerulus is jammed with lipid-filled cells, either histiocytic or endothelial. The mesangial cells, surrounded by a mesh of fibromucinous fibrils, are particularly well seen in the lower mid-portion of the field. Silver methenamine, hematoxylin and eosin stains. × 1,020.



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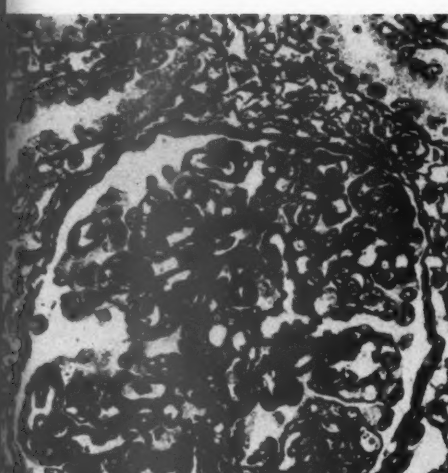

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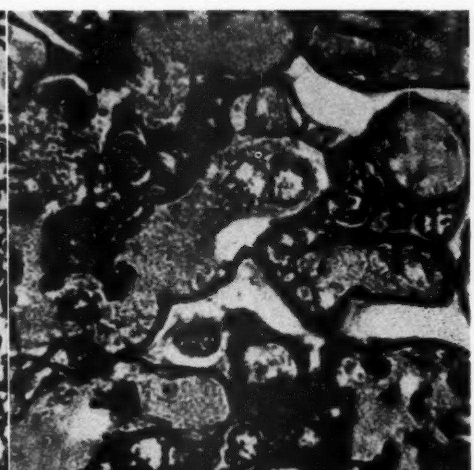

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FIG. 7. Nephrotic glomerulonephritis; chronic lobular lesion. Hematoxylin and phloxine stain. × 385.

FIG. 8. Nephrotic glomerulonephritis; chronic lobular lesion. Silver methenamine, hematoxylin and eosin stains. × 440.

FIG. 9. Nephrotic glomerulonephritis; membranous lesion. Of note are the thick capillary walls. Hematoxylin and phloxine stain. × 345.

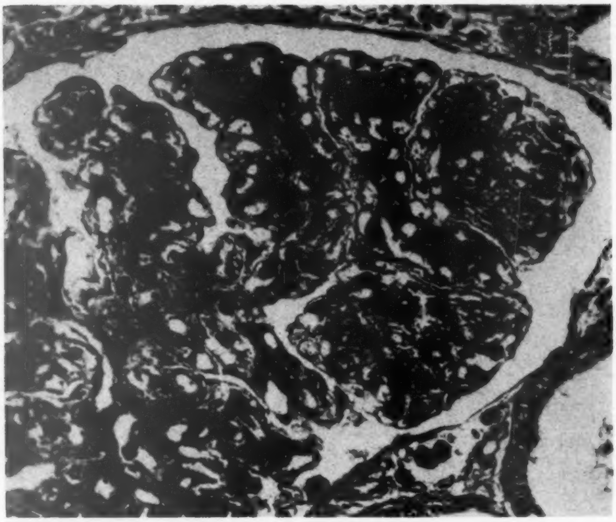

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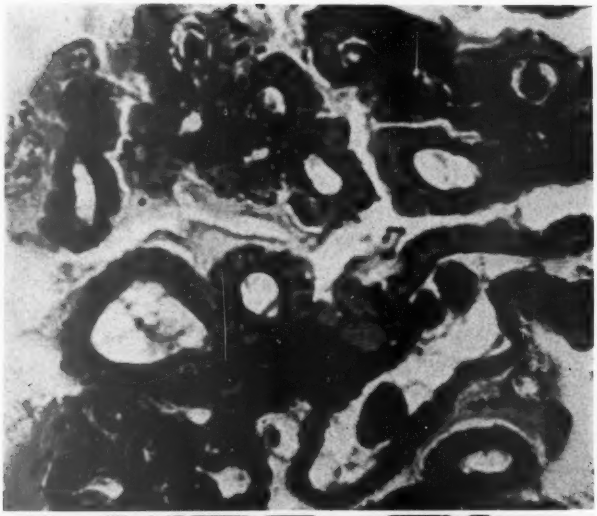
FIG. 10. Nephrotic glomerulonephritis; membranous lesion. The silver-positive clubs separated by hyaline droplets may be seen only fairly well since they are difficult to photograph. Silver methenamine, hematoxylin and phloxine stains. × 1,110.

FIG. 11. Nephrotic glomerulonephritis; membranous lesion. This is the advanced stage of hyalinosis in which only fragments of silver-positive material remain. Silver methenamine, hematoxylin and eosin stains. × 1,110.



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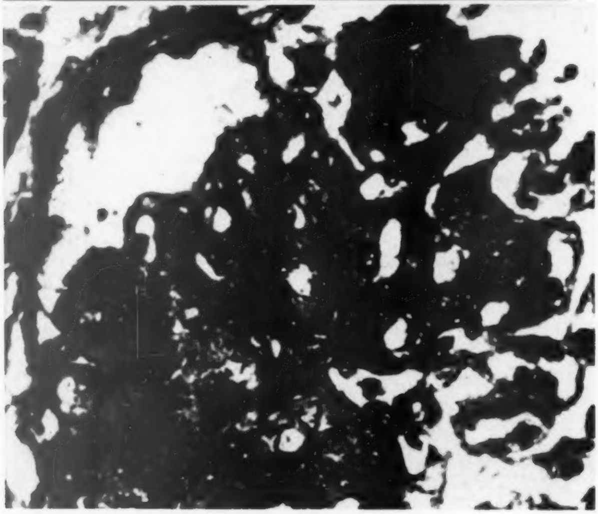

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HEPATIC LESIONS IN SICKLE CELL ANEMIA*

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Since 1923 when Sydenstricker *et al.*[1] made the first post-mortem study of sickle cell anemia, numerous reports concerning the pathologic findings of fatal sickle cell disease have appeared in the literature. The lesions of the liver rarely have been emphasized, although several investigators have commented on the incidence of palpable enlargement of the liver in fatal cases. It is conceivable that, because of the numerous vascular anastomoses in the liver, the lesions resulting from marked vascular and sinusoidal engorgement with sickled red blood cells have special significance.

Thirty-one cases of sickle cell anemia from the files of the Division of Pathology and Microbiology, University of Tennessee, which were necropsied during the years 1935 to 1955, have been studied. The ages of the patients range from 3 months to 45 years. Eighteen patients were under 20 years of age; the remaining 13 were 24 to 45 years of age.

Illustrative Case Histories

Case 1

A 27-year-old Negro male was admitted to the hospital with the chief complaints of severe joint pains mainly involving the knees and ankles and jaundice of approximately 4 weeks' duration. Epistaxis existed for 1 week prior to admission. Physical examination on admission revealed a jaundiced male with temperature of 104° F.; pulse, 108; respirations, 24; blood pressure, 120/80 mm. of Hg. An examination of the chest revealed minimal cardiac enlargement and the lungs were resonant throughout. The spleen was not palpable. The liver extended 5 cm. below the right costal margin and was slightly tender on palpation. No masses were felt in the abdomen.

The blood on admission gave a red cell count of 2.35 million per cmm., a hemoglobin of 7.5 gm. per 100 ml., and the peripheral blood smears revealed many sickled red cells, nucleated red cells, numerous target cells, diffuse basophilia with moderate anisocytosis and poikilocytosis. The white blood cell count was 33,500 per cmm. with a differential count of 12 per cent band forms, 75 per cent segmented forms, 6 per cent lymphocytes, and 7 per cent plasmacytes. The blood serum was icteric with a total serum bilirubin of 17.3 mg. per 100 ml. and a direct reaction of 8.6 mg. per 100 ml. Thrombocytes varied from 33 to 77 per 100 oil-immersion fields. The total serum protein was 6.2 gm. per 100 ml.; albumin, 2.8 gm.; globulin, 3.4 gm. The non-protein nitrogen of the blood was 28 mg. per

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100 ml. A cephalin flocculation test was 3 plus at 24 hours. The blood cultures and the serologic test for syphilis were negative. The urinalysis revealed neither sugar nor protein, with a specific gravity of 1.017, bilirubin 4 plus, and a trace of urobilinogen. No hemosiderin granules were seen but a few white blood cells and numerous red blood cells were noted.

The patient was given 1,500 cc. of glucose intravenously, antibiotics, vitamins, and electrolytes. After 24 hours in the hospital he went into shock, failed to respond to 1,500 cc. of whole blood, and died.

Necropsy was performed 3 hours after death. The body was markedly icteric. Approximately 500 cc. of clear fluid were removed from the thoracic cavities. The heart weighed 370 gm. and a slight fibrinous exudate covered the pericardial surface. The lungs were heavy, deep red, and firm. The liver weighed 2,450 gm. and was a deep reddish brown with a nodular external surface. On cut section the nodules varied considerably in size, being separated by broad bands of fibrous tissue which were distributed irregularly throughout; the largest nodule was 1.5 cm. in diameter. The spleen was very small, weighing 4.2 gm., and was extremely fibrotic. There was an ulcer with sharp edges in the duodenum. The gallbladder, the extrahepatic biliary tract, and other organs were within normal limits.

Microscopically, the hepatic sinusoids were distended and practically occluded by the stagnation of the sickled erythrocytes. There were associated large areas of recent necrosis in the paracentral zones (Fig. 1). Focal necrosis was observed elsewhere without any constant lobular relationship. Many pigment-containing macrophages were seen in the necrotic areas in which the sinusoids were occluded by networks of interlacing sickle cells (Fig. 2). The reticulum appeared to be intact in the areas of recent necrosis. Fibrous proliferation was demonstrated in old areas of necrosis in the peripheral and paracentral zones. In the portal areas there was massive deposition of connective tissue that penetrated into the lobules and separated the hepatic cells (Fig. 3). A moderate degree of bile duct proliferation was seen in the scar tissue. The bile canaliculi were distended with bile, but no evidence of biliary obstruction was noted either in the intrahepatic or extrahepatic system. No calculi were found.

Sections of the lungs revealed severe congestion and slight fibrosis of the alveolar septa. Many of the capillaries contained agglutinative thrombi composed of sickle cells. There were many pigment-laden macrophages in the alveoli. The splenic pulp was replaced by fibrous connective tissue, much of which had become calcified. The splenic capsule was also markedly fibrosed. Agglutinative thrombi composed of sickled red blood cells were observed in vessels of the kidney, pan-

creas, and duodenum. The glomerular tufts were adherent to the outer layer of Bowman's capsule without any inflammatory reaction and the glomerular capillaries contained thrombi.

Case 2

A 34-year-old Negro male was admitted to the hospital with severe abdominal pain and moderate jaundice. He had complained of abdominal cramps which led to the clinical suspicion of a ruptured peptic ulcer with peritonitis. However, the acute distress and the signs of peritoneal irritation subsided soon after admission. Physical examination on admission revealed a well developed male with an enlarged liver and spleen. The heart appeared to be normal in size and shape. The lungs were clear. The liver was 6 fingerbreadths below the right costal margin and nodular. There was slight tenderness in the right lower quadrant. The spleen was firm and readily palpable. Roentgenologic examination of the chest and the abdomen revealed no evidence of neoplasm or gastric ulcer. Peripheral blood smears revealed marked sickling of the red blood cells with marked variation in size, shape, and staining quality. Numerous diffusely basophilic cells, target cells, and a few Howell-Jolly bodies were noted. The red blood cell count was 2.2 million per cmm. and the hemoglobin 7.5 gm. per 100 ml. The hematocrit reading was 19.5 mm., and the non-protein nitrogen of the blood was 42 mg. per 100 ml. The white blood cell count was 16,700 per cmm. with a differential of 3.5 per cent band forms, 63 per cent segmented forms, 0.5 per cent eosinophils, 5 per cent basophils, 23 per cent lymphocytes, 4.5 per cent monocytes, and 0.5 per cent atypical cells. Total serum protein was 7.4 gm. per 100 ml. and consisted of 3.3 gm. of albumin and 4.1 gm. of globulin. A cephalin flocculation test was 4 plus at 24 hours. Urinalysis revealed neither glucose nor albumin.

The clinical impression was sickle cell anemia with an acute exacerbation. The patient was given large amounts of glucose intravenously, vitamins, morphine, and 500 cc. of whole blood. He died 2 days after admission.

Necropsy was performed 6 hours after death. The body was moderately icteric. Approximately 100 cc. of serosanguineous fluid was removed from the peritoneal cavity. No peritonitis or ruptured gastric ulcer was found. The heart weighed 325 gm. and there were no significant alterations in structure.

The liver weighed 2,910 gm. and was a reddish brown throughout. There were broad bands of fibrous tissue dividing the parenchyma into nodules of varying size (Fig. 4). The gallbladder and the intrahepatic and extrahepatic bile ducts were normal. The spleen weighed 350 gm. The capsule was thickened and the pulp was firm and dark red. The lymph nodes were enlarged and slightly firm. The remaining organs were within normal limits.

On microscopic study the hepatic parenchyma was irregularly divided by wide anastomosing bands of dense fibrous connective tissue. Heavy round cell infiltration was noted in the collagenous tissue. In the lobules the sinusoids were markedly distended and packed with large numbers of sickle cells. The associated central and paracentral

areas were necrotic. Marked fibrosis was noted in the paracentral necrotic areas. Large amounts of hemosiderin pigment were noted mainly within the liver cells, but a scant amount of iron-positive pigment was present in the Kupffer cells (Fig. 5). The scar tissue was free of pigmentation. Bile duct proliferation was limited. The broad fibrous bands contained many dilated veins occluded by sickle cells.

In the spleen were well circumscribed hemorrhagic infarcts containing numerous sickled red cells. The white pulp was extremely atrophic. A small amount of iron-positive pigment was identified. Subcapsular fibrosis was marked. The glomerular capillaries of the kidneys were markedly congested and their walls thickened. The basement membranes also were moderately thickened.

Case 3

A 15-year-old Negro boy was brought to the hospital after massive hematemesis with circulatory collapse. He had been admitted 7 years previously with the diagnoses of meningitis and sickle cell anemia. The hemoglobin level at that time was about 8.0 gm. per 100 ml. and the sickle cell preparation was immediately positive. The bleeding time, clotting time, and platelet count, however, were normal. The liver was enlarged and firm with a questionably nodular surface. The spleen was readily palpable and firm. Following his recovery from meningitis, the white blood cell count was 7,250 per cmm. with a differential count of 12 per cent band forms, 55 per cent segmented forms, 1 per cent eosinophils, 1 per cent basophils, and 29 per cent lymphocytes and 2 nucleated red blood cells.

On the final admission the patient was in severe shock without detectable blood pressure, respirations, or heart sounds. There was a history of massive hematemesis on two previous occasions. The liver was palpable 3 cm. below the right costal margin and was nodular. The spleen was enlarged, firm, and nodular. The hematocrit reading was less than 10 mm., and the hemoglobin was less than 2.5 gm. per 100 ml. The white blood cell count was 40,000 per cmm. and segmented neutrophils predominated. After the administration of intravenous fluids, epinephrine, caffeine, oxygen, plasma, and 500 cc. of whole blood, the blood pressure rose to a maximum of 96/50 mm. of Hg. The heart rate was 72, and respirations were slow and irregular. Response to treatment was transitory and death occurred 6 hours after admission.

Necropsy was performed 1 hour after death. The sclerae were icteric. The liver weighed 1,700 gm. and was extremely nodular and firm (Fig. 6). The parenchyma was separated into nodules, which varied in size, by broad ribbons of gray fibrous tissue. The largest nodule was 4 cm. in diameter and was dark red. The gallbladder and the extrahepatic and intrahepatic bile ducts were normal. Material was taken from the liver for the culture of Brucella and other organisms. The spleen weighed 900 gm., and consisted of a firm, dark pulp in which were numerous discrete nodules. Both lungs were heavy and hemorrhagic. Varices were present in the lower portion of the esophagus. The heart and the remaining organs were within normal limits.

Microscopically, the hepatic lobules were irregularly penetrated by strands of connective tissue accompanied by a heavy round cell infiltration and slight bile duct proliferation. The sinusoids were extremely dilated and occasionally packed with sickle cells. In some areas patchy, recent necrosis involved the peripheral zones. The central veins could not be definitely identified. Arteries and veins were occluded by agglutinative sickled red blood cells. In the adjacent areas there was a marked separation of parenchymatous cells and round cell infiltration (Fig. 7). The sickle cells in some of the distended veins were arranged perpendicularly to the vessel wall (Fig. 8). A few hepatic arterioles were occluded also by agglutinative thrombi. Small amounts of iron-positive pigment were noted only in the hepatic cells. The reticulum was collapsed in the necrotic areas (Fig. 9).

Large, well circumscribed, hemorrhagic areas in the spleen were separated by atrophic white pulp. A moderate amount of iron-positive pigment was present but there was no fibrosis. The glomerular capillaries were packed with sickled red blood cells. The tubular epithelial cells contained small amounts of iron pigment. Agglutinative thrombi were observed in the vessels of the lungs, kidneys, and pancreas.

Case 4

A 35-year-old Negro male farmer was admitted to the hospital complaining of pain in both feet, in the para-umbilical area, left thigh, and back of the head for approximately 2 weeks. Three weeks before admission the onset of a "cold and fever" was accompanied by persistent productive cough. During this period, weakness and dizziness had kept him from work. Two years previously he had suffered a torsion injury of the left knee.

On admission his temperature was 100° F.; pulse, 85; respirations, 28; blood pressure, 125/65 mm. of Hg. There were discrete, non-painful, enlarged posterior cervical and supraclavicular lymph nodes. The veins of the neck were moderately distended, and the conjunctivae and mucous membranes were markedly icteric. There was a systolic murmur at the cardiac apex. The liver was palpable 4 cm. below the right costal margin and questionably nodular. The spleen was not palpable. There was atrophy of the lower part of the left thigh.

The hemoglobin was 7.5 gm. per 100 ml. The red blood cell count was 1.2 million per cmm., and the white blood cell count was 41,650 per cmm. with a differential count of 72 per cent segmented forms, 25 per cent lymphocytes, and 3 per cent monocytes. The total serum protein was 7.7 gm. per 100 ml.; albumin, 3.5 gm.; globulin, 4.2 gm. The total serum bilirubin was 64.5 mg. per 100 ml. and the 1 minute value was 18.9 mg. per 100 ml. Fifty-one platelets were counted in 100 oil-immersion fields. The four-tube clotting time was 62 minutes and no clot retraction was observed in 4 hours. The upper part of the clot consisted of deeply icteric material resembling chicken fat. The non-protein nitrogen of the blood was 44 mg. per 100 ml. Urinalysis revealed 2 plus protein; sugar, negative; specific gravity, 1.014; pH, 7.5. The urine was clear and dark, resembling the color of Coca Cola. The foam test for bilirubin was positive, and urobilinogen was positive at 1:40 dilution. The preparation for sickle cells was immediately positive. Roent-

genologic examination of the abdomen revealed generalized osteoporosis and accentuation of the trabecular pattern of the lumbar vertebrae, iliac wings, and femur. The patient was critically ill during the entire period in the hospital and had repeated nasal hemorrhages. One thousand cc. of whole blood, vitamin K, calcium gluconate, and glucose solutions were administered. Bleeding from the nose, throat, and gastro-intestinal tract continued and the patient expired on the eighth hospital day.

Necropsy was performed 4 hours after death. There was obvious icterus and the peritoneal cavity contained about 400 cc. of a yellow translucent fluid. No fluid was found in the pleural cavity. The heart was slightly enlarged owing to some hypertrophy of the left ventricle. Numerous petechiae were scattered throughout the epicardium. In the tips of the papillary muscles dark purple areas of discoloration and calcification were noted. The lungs weighed 640 gm. each and were moderately edematous. The spleen weighed 30 gm. and was markedly fibrosed; the architectural markings were lost. Both kidneys were swollen and deeply stained with bile.

The liver weighed 2,250 gm. The entire organ was a deep mahogany color, firm and moderately nodular. The cut surface showed a definite increase in fibrous tissue which separated the parenchyma into nodules of varying size. The gallbladder was dilated but contained no stones. The extrahepatic and intrahepatic bile ducts were normal. Throughout the gastro-intestinal tract a considerable amount of blood was present. The veins of the lower portion of the esophagus were markedly dilated but no point of rupture was found.

On microscopic examination the hepatic lobules were partially replaced by very broad masses of fibrous scar tissue in which there were numerous round cells and many proliferated bile ducts. These fibrocollagenous bands separated the parenchyma into nodules of varying size. The sinusoids were extremely distended and filled with agglutinative sickle cells. In a few peripheral areas the necrotic hepatic cords were replaced by fibrillar tissue (Fig. 10). The majority of the hepatic cells were heavily pigmented. A few of the hepatic veins were obliterated by agglutinative thrombi.

Sections of the spleen revealed no recognizable splenic architecture, only dense hyalinized fibrous tissue containing numerous calcific particles. The capillaries of the alveolar walls of the lungs were extremely dilated and filled with sickle cells. The glomerular capillaries contained agglutinative thrombi and a few glomeruli were partially fibrosed. There was pigmentation of the renal tubular epithelium. The structure of the lymph nodes was partially destroyed by severe hemorrhage.

Case 5

An 11-year-old Negro girl was admitted to the hospital with the chief complaint of pain in the epigastric area for 24 hours. The pain had commenced in the left lower quadrant, radiated to the epigastrium, and persisted. There was no nausea or vomiting. The patient noted edema the day before admission. There was no family history of sickle cell anemia. Physical examination on admission revealed temperature of 101° F.; pulse, 88; respirations, 22 per minute. There was bleeding from the upper gums. The sclerae were icteric. The heart was not remarkable and the lungs were resonant. The spleen was not palpable. The liver was enlarged and slightly tender. The skin of the lower extremities was ulcerated. Roentgenologic examination of the chest revealed calcified hilar lymph nodes but the lung fields were clear.

The red blood cell count was 1.75 million per cmm. with a hemoglobin of 6 gm. per 100 ml. The red blood cells showed marked anisocytosis and poikilocytosis. Numerous sickled cells were seen in a peripheral blood smear. White blood cell count was 17,500 per cmm. and a differential count included 1 per cent eosinophils and 16 per cent blast forms. The urine was dark amber and without sediment; albumin, sugar, and acetone were negative. The specific gravity was 1.013. Serologic test for syphilis was negative. The clinical impression was an acute exacerbation of sickle cell anemia. The patient continued to complain of severe abdominal pain, and the heart rate remained rapid. She died 6 days after admission.

Necropsy was performed 2 hours after death. There was obvious icterus of the sclerae. The heart weighed 240 gm., and both ventricles were dilated; no valvular lesions were noted. The lungs weighed 200 gm. each and were grossly consolidated. The spleen weighed 35 gm. and was markedly fibrosed. The liver weighed 1,740 gm., and was dark red. The surface was irregular and a few nodules projected from the capsule. On cut section the organ was very firm and islands of parenchyma were surrounded by fibrillar tissue. The intrahepatic and extrahepatic bile ducts, the gallbladder, and other viscera appeared normal.

Numerous microscopic sections of the liver revealed distended sinusoids occluded by hemolyzed sickle cells (Fig. 11). The central and paracentral areas showed necrosis and a thickened reticulum. The fibrous connective tissue, which separated the hepatic cells in the paracentral necrotic areas, contained a moderate number of round cells and a few eosinophils. There was a moderate degree of pigmentation of the hepatic cells. The Kupffer cells were not remarkable.

The spleen showed marked siderofibrosis with slight calcification. The lungs revealed interstitial pneumonia but no thrombi. There was slight pigmentation of the renal tubular epithelium. There were no significant lesions in the remaining viscera, including the gastrointestinal tract.

Case 6

A 14-year-old Negro boy was admitted to the hospital on May 22, 1945, and died 8 days later. From early infancy until his death, he had been subject to attacks of febrile illness with rheumatic pains. The sclerae were icteric and the urine dark, and during the periods of sickness, jaundice became more marked. Eight years prior to the final admission the patient was admitted with the complaints of priapism and inability to void.

Physical examination on the last admission revealed a temperature of 99° F.; pulse, 84; respirations, 24; slight generalized lymphadenopathy; hypertrophied tonsils; a slightly enlarged heart with a soft systolic murmur; a distended abdomen; and a firm erect penis which was painful when touched. The spleen was not palpable. The muscles were poorly developed. The liver extended 3 fingerbreadths below the right costal margin. A red blood cell count was 2.15 million per cmm.; hemoglobin was 6.5 gm. per 100 ml.; the white blood cell count was 22,400 per cmm. and showed an essentially normal differential count. The routine urinalysis was negative. The urologist's examination revealed a hard swelling just back of the glans penis and general engorgement of the vessels. A large needle inserted in the corpora cavernosa failed to drain them. Circumcision was performed, following which the child was slow to regain consciousness. Engorgement of the penis was less, but pain continued. Five days after the procedure the child became dyspneic and complained of pain in his right chest. Cough and signs of consolidation of the right upper lobe were present. Sulfapyridine was given without improvement. The clinical impression was sickle cell anemia and pneumonia of the right lung with cardiac enlargement. The patient died 7 days after the operation.

Necropsy was performed 9 hours after death. The body was that of a poorly developed male child with obvious icterus. The heart weighed 220 gm., and was diffusely enlarged. The right auricle and ventricle were dilated, but the valves were not unusual. The cardiac muscle appeared normal. The right lung weighed 420 gm. and was markedly consolidated; the left lung weighed 320 gm. and was extremely congested. The spleen weighed 8 gm. and was very firm and dark purple. The kidneys were extremely congested. The liver was mahogany red, weighing 1,700 gm.; the surface was relatively smooth. The cut sections revealed an increase in fibrous tissue with a nodular configuration. The bile ducts were not prominent. The gallbladder contained a few brownish stones.

On microscopic examination of the liver, agglutinative thrombi composed of sickle cells were found in the veins of the portal areas. The sinusoids were distended and were partially occluded by hemolyzed sickled red blood cells. In a few areas the hepatic cells appeared to be floating in pools of sickled cells. There were many areas of focal necrosis without any definite relation to the lobular pattern. The periportal fibrous tissue was irregularly distributed, separating groups of hepatic cells. There was moderate round cell infiltration. Golden yellow granules of pigment which gave a positive reaction for iron were present in the hepatic cells. Bile duct proliferation was rarely ob-

served in the scar tissue. In the hepatic lobules, the central veins were occluded by thrombi consisting of numerous sickled red blood cells and polymorphonuclear leukocytes.

The spleen was markedly fibrosed and contained a large amount of iron-positive pigment. In the sections of the kidneys, sickled red blood cells almost completely filled the space between the layers of Bowman's capsule. The cells of the convoluted tubules were filled with yellow-brown to black granules. Bronchopneumonia involved the entire upper lobe of the right lung. Many of the pulmonic veins of the left lung were occluded by agglutinative thrombi and there was extensive intra-alveolar hemorrhage. Sections of the corpora cavernosa revealed thrombosis of the vessels and extreme engorgement. A small ulcer was demonstrated in sections of the skin of one leg. The lymph nodes were extremely congested.

Review of Pathologic Findings

Size and Color of Liver. Grossly, the livers were consistently enlarged and a deep purple or mahogany red. Similar changes were described by Corrigan and Schiller,[2] who stated that the enlarged livers varied from deep brown to deep purple. Lowe and Adams[3] commented that the hepatomegaly probably was due to stagnation of sickled red blood cells in the hepatic sinusoids, and Lash[4] reported that the portal capillaries were engorged with sickle cells in the enlarged liver of his case.

Stagnation of the Sickled Erythrocytes in the Hepatic Sinusoids. The most prominent and constant microscopic feature of all of the cases was severe distention of the sinusoids by sickled red blood cells. In a few of our cases isolated hepatic cells appeared to be floating on pools of sickle cells and had no connection with the hepatic cords. Similar changes were mentioned by Green *et al.*,[5] who described congestion of the sinusoids by sickle cells and widespread phagocytosis of large numbers of red blood cells by the Kupffer cells. Ryerson and Terplan[6] presented 2 cases of fatal sickle cell disease in which the hepatic lobules were obscured by marked sinusoidal dilatation and the hepatic cells were moderately degenerated. In our series the sinusoids frequently were occluded by masses of sickled red blood cells forming a fibrin-like network as illustrated by Figure 2.

Phagocytosis of erythrocytes by the Kupffer cells in the engorged sinusoids was not observed frequently in our cases, although Jaffé[7] noted that the Kupffer cells were generally swollen, contained prominent granules, and showed phagocytosis of sickled red blood cells.

The Kupffer cells observed in the present series were slightly swollen but otherwise not remarkable.

Hepatic Necrosis and Atrophy. Necrosis and atrophy were most severe in the paracentral areas as illustrated by Figure 1, and degenerative changes were less severe in the central zones. In practically all cases there was marked sinusoidal stagnation of the sickle cells and varying degrees of atrophy and focal necrosis of hepatic cells. In 6 cases a more widespread recent necrosis was present which showed no constant relationship to either central or peripheral regions. Fibrous proliferation was confirmed by special stains in some of the necrotic areas while the other areas showed a relatively intact reticular network. Similar findings were observed by Heilbrun[8] and Hamman,[9] who described marked atrophy of the hepatic cells with pigmentation, extreme dilatation of sinusoids, and round cell infiltration about the portal areas.

Degree of Pigmentation. The degree of hemosiderosis was variable and not related to the duration of the disease in our cases. In most livers, hemosiderin as well as other pigments was found primarily in the hepatic cells and only rarely in the Kupffer cells and scar tissue. A severe degree of hemosiderosis was observed in a 5-year-old child whose hepatic cells contained a tremendous amount of iron-positive pigment. On the other hand, a 32-year-old patient who had received a large amount of whole blood had a minimal degree of pigmentation of hepatic cells but many pigment-laden macrophages were present in the necrotic regions. Sydenstricker *et al.*[1] reported marked pigmentation of the hepatic cells with a considerable degree of atrophy in a 5-year-old male child.

Cholecystitis and Cholelithiasis. Chronic cholecystitis and cholelithiasis were observed in 2 cases. The extrahepatic and intrahepatic biliary tracts were normal and showed no developmental anomalies. In four instances bile plugs were observed in the dilated bile canaliculi, but there was no evidence of obstruction in the biliary system.

Occlusion of the Sinusoids by Intrasinusoidal Hemolysis. In 7 of our cases the hepatic sinusoids were partially occluded by hyaline thrombi, as illustrated by Figure 11. Green *et al.*[5] described similar findings in 2 of their cases, hyaline fibrin-like thrombi of unknown cause. Our investigation suggests that they probably were caused by the hemolysis of sickle cells.

Thrombi. Agglutinative thrombi in the hepatic arterioles and venous capillaries, as illustrated by Figures 7 and 8, were observed in six instances, or 20 per cent of the cases. A central vein was occluded

by an agglutinative thrombus in one case. The hepatic capillaries in the portal areas were most frequently occluded by thrombi. In case 3, many veins were occluded by agglutinative thrombi and this resulted in marked separation of hepatic cells with round cell infiltration, as illustrated by Figure 7. The thrombosed veins were distended and contained many sickled red blood cells arranged perpendicularly to the vascular walls.

In their report of a case, Ching and Diggs[10] described agglutinative thrombi in hepatic capillaries around which heavy round cell infiltration was apparent. Crastnopol and Stewart[11] reported on liver biopsies in patients with sickle cell anemia, and stated that the hepatic capillaries were occluded by agglutinative thrombi and that the lesions were sufficient to establish a diagnosis of chronic hepatitis. Kimmelstiel[12] described many areas of hepatic necrosis surrounded by narrow hemorrhagic zones in a fatal case of the disease in an 11-year-old girl. No vascular thrombi were noted by him.

Patterns of Fibrous Proliferation. Seven of 31 cases (2 children, 5½ and 9 years of age, and 5 adults) showed a considerable amount of fibrosis in the peripheral zones, which probably represented healed massive necrosis as illustrated by Figure 10. The patterns of fibrous proliferation were irregular, and bands of tissue penetrated all regions of the lobules. Those livers which showed necrosis in the central and paracentral zones also revealed increased amounts of connective tissue in the necrotic areas. Similar changes were described previously by Steinberg,[13] who presented 7 cases of sickle cell anemia in which the livers showed increased connective tissue in the central and paracentral lobular zones, as well as an increase in the periportal connective tissue. Steinberg's observation is confirmed by our study. Marked fibrosis associated with hemosiderosis was reported by Tomlinson.[14] Fibrosis of diffuse type was mentioned previously by Hargrove and Mathews,[15] who presented a case of hemochromatosis in which the cells were extremely pigmented and diffuse fibrosis was present. Round cell infiltration was seen throughout the tissue.

Cirrhotic Changes. Nine of the 31 patients had advanced cirrhosis. Three of them were under the age of 15 years and gave no history of blood transfusions prior to admission to the hospital. One of the cirrhotic livers showed massive deposits of iron-positive pigment in the liver cells and small amounts of pigment in the Kupffer cells, but the scar tissue was free from pigmentation as illustrated by Figure 5.

The gross appearance of the liver illustrated by Figures 4 and 6 suggested a macronodular or a postnecrotic cirrhosis. Many broad

bands of loose fibrillar material penetrated the atrophic lobules. Foci of recent necrosis were observed in these cases. Dale[16] and Graham[17] presented examples of chronic hepatitis in sickle cell anemia which showed extensive cellular degeneration and fine, patchy, irregularly distributed fibrous scars with lymphocytic infiltration. Ryerson and Terplan[6] observed similar changes and suggested the term subacute toxic dystrophy. Legant and Ball[18] reported a case of hepatic cirrhosis in fatal sickle cell disease and also cited 4 other cases which showed extensive degeneration and massive necrosis of the hepatic cells. None of these patients gave a history of infectious hepatitis or other specific infectious disease. Green *et al.*,[5] in their extensive analysis of 21 cases of fatal sickle cell anemia, reported 4 examples of cirrhosis.

Discussion

The lesions of the liver in sickle cell disease appear to be related to various mechanisms: (1) long-standing severe anemia, (2) a prolonged hemolytic process with increased excretion of bilirubin and deposition of pigment, (3) repeated blood transfusions which might lead to exogenous hemochromatosis, (4) stagnation of sickled red blood cells in the sinusoids with sinusoidal obstruction, (5) vascular occlusion by agglutinative thrombi.

It is conceivable that prolonged severe anemia may contribute to the hepatic damage. However, a pronounced degree of hepatic damage is rarely observed in patients with chronic anemia, such as congenital spherocytic or hemolytic anemia. Patients with anemia may show cardiac hypertrophy or dilatation, and chronic congestive cardiac failure could account for the hepatic lesions. In our series, marked cardiac dilatation was observed frequently. No evidence of congestive cardiac failure was noted for the patients although a few showed electrocardiographic alterations. Usually, the liver became enlarged and firm prior to the development of cardiac enlargement.

According to Higgins,[19] the heart is enlarged in 72 per cent of patients with sickle cell anemia, but correction of the anemia frequently results in a return of the heart to normal size and a disappearance of the murmurs. Klinefelter[20] pointed out that the cardiac hypertrophy and dilatation compensate for the prolonged anoxemia in patients with sickle cell anemia, and that congestive cardiac failure, which does not respond to digitalis, is seen only in the terminal stages of the disease.

The prolonged hemolytic process with pigment deposition and increased excretion of bilirubin could provoke a biliary tract disorder

with the formation of calculi or the stagnation of bile in the liver. Chronic cholecystitis and cholelithiasis were observed in only 2 cases, and the biliary tract in the remaining cases was not remarkable. It seems clear from our study that the hepatic damage in sickle cell anemia cannot be attributed solely to the anemia or to the hemolytic process.

Repeated blood transfusions in patients with chronic anemia may lead to liver damage by serum hepatitis or by the production of exogenous hemochromatosis. There was no clinical history indicating that any of the patients developed serum hepatitis from blood transfusion. The liver was enlarged prior to any transfusion in most of our cases. One child 9 years of age had received six blood transfusions, the last having been administered 5 days before death. At necropsy, the liver showed broad bands of loose fibrillar material penetrating atrophic lobules, with foci of recent necrosis and a small amount of iron-positive pigment in the hepatic cells. The Kupffer cells and the scar tissue were not pigmented. No correlation between hepatic lesions and transfusion reaction could be established. The cirrhosis in 9 instances was thought not to be due to transfusions.

The serologic tests for syphilis, when done, were negative. No clinical or pathologic evidence of syphilitic infection existed. Severe dietary deficiency was not of significance in our series.

The sinusoidal stagnation of sickled red blood cells and the agglutinative thrombi in the liver seemed to be responsible for necrosis by blocking the blood flow and reducing the blood supply. Hyaline thrombi caused by intrasinusoidal hemolysis also impaired the circulation and resulted in necrosis. Kimmelstiel[12] described massive necrosis of the liver in a young girl who had had no transfusions and in whose liver there was no evidence of vascular occlusion. He assumed that the lesions were the result of acute vascular spasm. Theoretically, vascular spasm might produce a massive infarct but no such lesions were found in our series.

Summary and Comment

Thirty-one necropsies performed on patients who had died of proved sickle cell anemia were reviewed. Morphologic evidence of hepatic cell damage was noted in all cases, and in 9 cases cirrhosis was found. Histologic study of these livers suggested that the lesions in sickle cell anemia were caused by an impairment of blood flow resulting in an anoxic necrosis of the hepatic cells. Sinusoidal blockage by stagnation of the sickled red blood cells, or by hyaline thrombi caused

by intrasinusoidal hemolysis, and the vascular agglutinative thrombi of the hepatic capillaries appeared to be responsible for the anoxic necrosis.

In the cirrhotic livers the changes appeared to be of a macronodular or a postnecrotic type. This type of cirrhosis seems to be a unique manifestation of fatal sickle cell disease.

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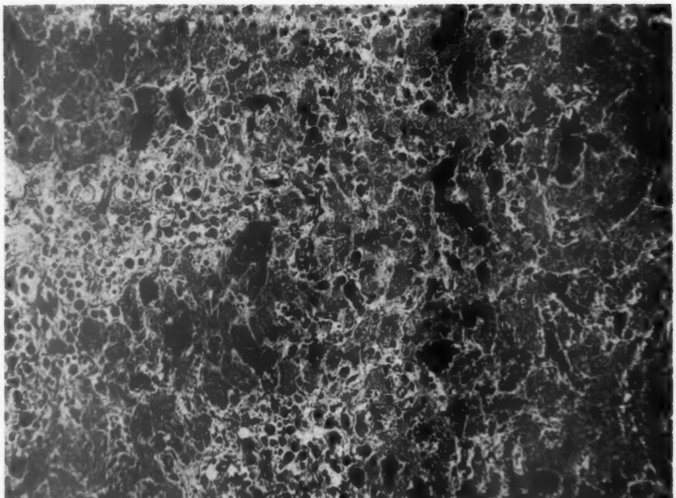

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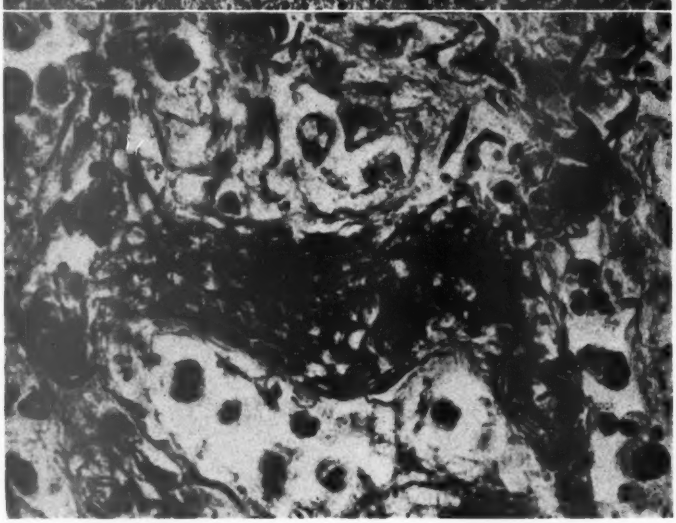

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LEGENDS FOR FIGURES

FIG. 1. Case 1. Large area of necrosis. Masson's trichrome stain. × 160.

FIG. 2. Case 1. High-power view of necrotic area shown in Figure 1. Anoxic necrosis is caused by sinusoidal obstruction which is due to the formation of a network of sickled red cells. Masson's trichrome stain. × 430.

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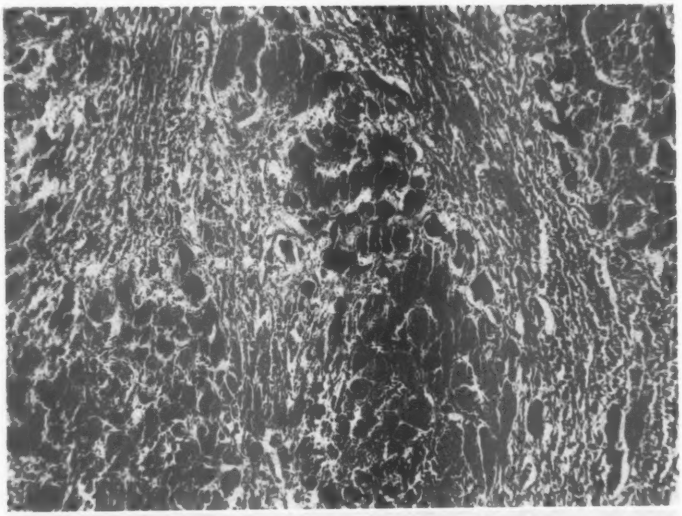

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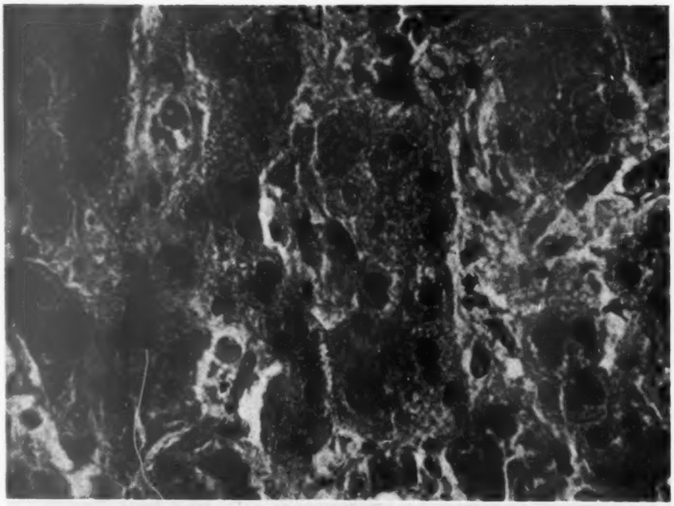
FIG. 3. Case 1. Bands of connective tissue penetrate the hepatic lobules. Masson's trichrome stain. × 330.

FIG. 4. Case 2. Gross picture of the liver. Grayish fibrillar material divides the hepatic parenchyma into nodules of various size.



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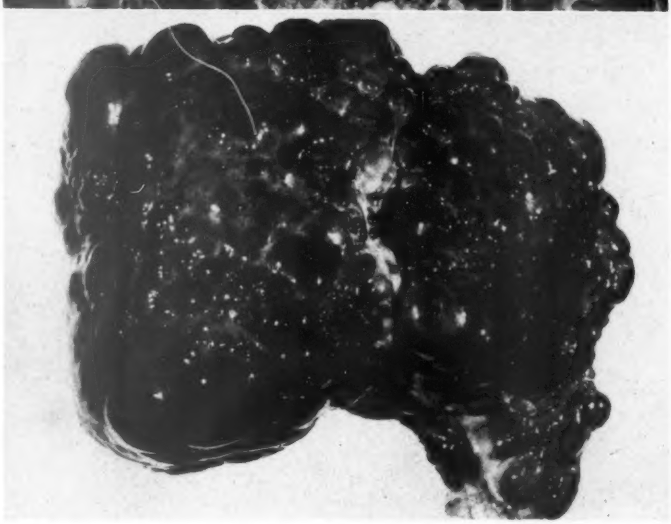

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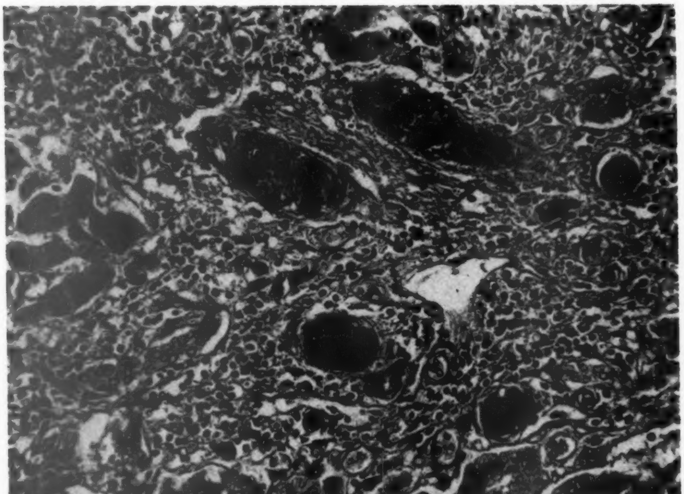
FIG. 5. Case 2. Hemosiderin pigment within the liver cells. Prussian blue stain × 430.

FIG. 6. Case 3. Cirrhosis of macronodular type.

FIG. 7. Case 3. A group of vessels is occluded by agglutinative sickle cells. Marked fibrosis and round cell infiltration about the vessels may be noted. Masson's trichrome stain. × 160.

FIG. 8. Case 3. High-power view of thrombosed vein. Masson's trichrome stain. × 460.



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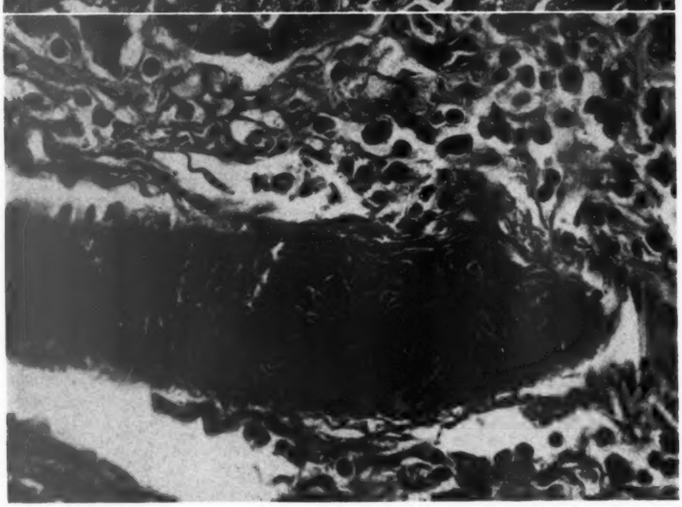

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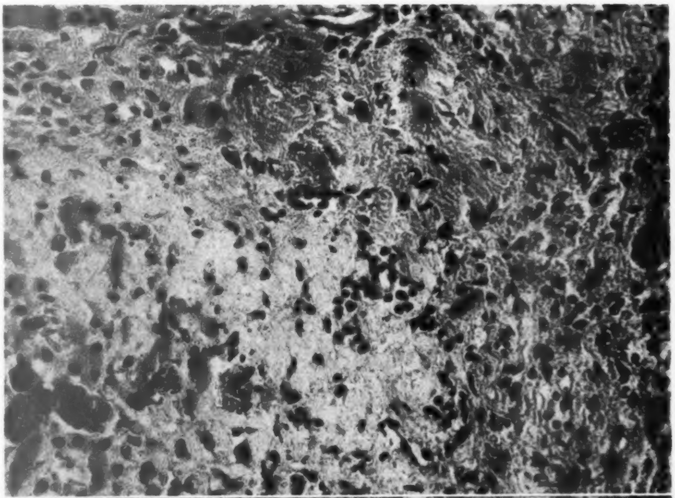
FIG. 9. Case 3. Thickened and partially destroyed reticular structures in the necrotic area. Reticulin stain. × 430.

FIG. 10. Case 4. Fibrous scar tissue in the peripheral zone. Hematoxylin and eosin stain. × 360.

FIG. 11. Case 5. The hepatic sinusoids contain a homogeneous material caused by hemolysis of sickled red blood cells. Hematoxylin and eosin stain. × 430.



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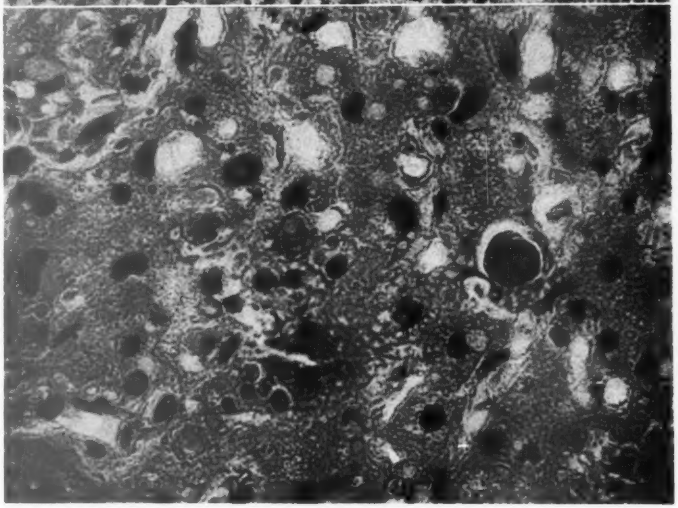

11

THE PATHOLOGY OF GOSSYPOL POISONING*

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The toxic effects of prolonged ingestion of cottonseed meal were recognized in animals as early as 1911.[1] Since this substance is produced in plentiful quantities as a by-product of the preparation of cottonseed oil, and since it is in other respects a valuable and concentrated source of protein for the feeding of livestock, its use for that purpose has continued and the limits of safety in the rations of domestic animals have received extensive study. Gossypol was reported to be the toxic component of cottonseed in 1916,[2] but for many years this was not universally accepted. One cause of confusion was the frequent concomitance of vitamin A deficiency in the diets which were used both commercially and experimentally, so that the claim persisted in some quarters that cottonseed poisoning was equivalent to avitaminosis A. It can now be accepted that gossypol is toxic to an important degree, especially in swine, which are more susceptible to it than other species.

The extraction of the oil from cottonseed is accomplished by several different processes. The gossypol remains in the meal and the processor endeavors to keep it at a level approximating 0.02 to 0.04 per cent but, owing to variations in temperature when processed by the hydraulic-press and to undesirable solvents in other processes, this amount may be greatly exceeded.

Our diagnostic routine afforded the opportunity to perform necropsies upon, and to examine the tissues of, pigs that died in the course of an experiment on the feeding of cottonseed meal which was conducted under the direction of Professors Fred Hale and Carl M. Lyman of the Animal Husbandry and Biochemistry Departments, respectively, of the Agricultural and Mechanical College of Texas. The details of this and other experiments in the feeding of cottonseed meal will be reported by Professors Hale and Lyman elsewhere. Since opportunities to study the effects of gossypol under accurately known and precisely controlled conditions are not numerous, and since pathologic criteria for the diagnosis of fatal poisoning have been questioned, it is desired to describe in this paper the pathologic changes encountered in 18 pigs which died in the course of the experiment.

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Methods

The pigs were fed a diet provided with adequate amounts of all known essential nutrients, including vitamin A. Cottonseed meal of previously determined gossypol content was added so that different lots of pigs received diets which contained free gossypol in amounts ranging from zero to 0.03 per cent of the total ration. The animals that died (the subjects of this report) came from lots receiving from 0.02 to 0.03 per cent. In accord with the usual experience of the above-named investigators, amounts of free gossypol smaller than 0.01 per cent were invariably harmless; amounts between that figure and 0.02 per cent seldom produced specific symptoms but did lead to unsatisfactory growth.[3]

Two of the pigs on 0.01 per cent of free gossypol were studied in detail as controls for comparison with the 18 which died. After 70 days on this level of gossypol, during which they consumed respectively 22 and 24 gm. of gossypol and made excellent growth, these pigs were slaughtered for food, all organs being normal grossly and microscopically.

Results

As shown in Table I, the animals that died had been on the gossypol ration from 38 to 79 days, with the exception of 2 which survived until the 93rd day. It should be understood that even the highest level of gossypol used was not necessarily lethal, considerable numbers of pigs showing only retarded growth when marketed at the usual age of 6 to 8 months.

In the seriously poisoned animals signs of illness were typically apparent for 2 to 6 days, or, exceptionally, as long as 1 month. The outstanding symptom was always dyspnea, with violently labored respirations which stockmen

TABLE I

Animals Dying During the Experiment

Animal number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Number of days on gossypol	38	43	46	50	57	60	62	74	77	93	93	45	79	45	48	63	76	77
Gossypol in ration, per cent	0.03	0.03	0.027	0.03	0.02	0.0225	0.0225	0.027	0.027	0.027	0.0225	0.0225	0.0225	0.0225	0.028	0.0225	0.0225	0.028
Number of days sick	3	12	5	10	7	8	?	?	?	?	?	18	3	4	14	18	27	29

? = Information not available.

call thumping. Progressive weakness and emaciation were accompanied by a good appetite almost until death.

The gross and microscopic lesions in the 18 pigs are believed to be characteristic of cumulative poisoning by gossypol. As can be seen from Tables II and III, widespread congestion and edema were salient

TABLE II
Gross Pathologic Lesions

Animal number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Subcutaneous edema	+				+		+			+			+				+	+
Hydrothorax	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Congestion and edema of lungs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydropericardium	+	+			+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dilatation of heart	+	+	+	+	+	+	+	+	+	+		+	+		+			+
Hypertrophy of heart					+	+			+			+	+					
Hydroperitoneum	+			+	+	+	+	+	+	+		+				+	+	+
Edema of gallbladder		+	+		+	+	+	+	+				+					
Edema of lymph nodes	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+		+
Congestion (and necrosis?) of liver	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Congestion of kidney	+		+	+		+	+		+	+	+	+	+		+			+
"White muscles"	+	+			+	+	+	+	+	+	+							+
Icterus														+			+	+

features. The lungs and liver were markedly congested in all cases; the kidneys, spleen, and lymph nodes, in 75 per cent; and various other sites not included in the tables, such as the ventral belly wall, the adrenal and thyroid glands, frequently shared in the excess of venous blood.

Large amounts of fluid often were encountered in the pleural, pericardial, and peritoneal cavities. Edema of the lungs, present in all cases, was commonly so extensive that a frothy fluid was visible in the trachea. While the alveoli contained their share of fluid, microscopically much of the edema in the lungs was found in the interstitial tissues, the interlobular septa and subpleural layers being greatly distended by accumulated fluid. Edematous lymph nodes could usually be found in the body cavities, as well as in the cervical region and elsewhere. Subcutaneous edema listed in Table II involved the ventral abdominal wall or the distal parts of the limbs, or both, in certain animals. In addition to the sites included in Table II, divers other organs, such as the urinary bladder and thyroid gland, were frequently edematous. In the liver the separation of reticulum from hepatic cords which is usually interpreted as edema was observed frequently in

areas where it was not obscured by the more destructive changes to be described.

In accordance with accepted principles, the edema was considered to have been dependent on passive congestion and venous stasis. The latter conditions were attributed to a progressively failing heart on the basis of both symptoms and lesions. In all but 4 of the animals (77 per cent) the heart was conspicuously flabby and dilated. Degenerative changes consisting of partial dissolution (cytoplasmolysis)

TABLE III
Histopathologic Lesions

Animal number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Lung, congestion	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Edema	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Thickening of alveolar walls											+					+	+	
"Epithelioid" phagocytes		+		+	+	+				+		1		+			+	+
Alveolar hemorrhage																+	+	
Pneumonia of limited extent			+		+										+	2	+	+
Heart, degeneration	+	+	+			+	+	+	+	+	+		+			?	+	3
Hypertrophy	+	+	+			+	+	+	+	+	m	+	+	m	+	?	m	+
Intestine, enteritis				+			+							+	+			
Liver, severe congestion	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Loss of parenchyma	+	+	½	+	+	½	+	+	+	+	+	½	+	½	+	½	+	+
Spleen, congestion	?	+	+	+	+					+	+	+	+	+	+	?	+	+
Increased reticulo-endothelium	?		+		+	+								+		?		+
Atrophy	?		+		+	+	+		+	+		+	+		+	?	+	
Lymph node, edema	+	+	+	+	+	+	+	+	+	?	+	?	+	4	+	+	+	+
Congestion			+	+	+		+			?	½	?	+	4	+	+	+	+
Hemorrhage				+	+		+			?		?	+		+		+	+
Lymphoid atrophy			+				+	+		?	+	?					+	+
Kidney, congestion	?		+	+	+	+		+	+	+	+	+	+	+		+	+	+
Cloudy swelling (necrosis)	?		+	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Lipidosis	?				+			+	+			+						
Edema	?										+							

+ = Present and distinct.
½ = Present, but less marked than usual.
m = Minimal or doubtful.
? = No data available.
1 = Phagocytic reticulo-endothelial cells, not epithelioid in appearance.
2 = Chronic pneumonitis.
3 = Degeneration of myocardium in region of an inflammatory endocardial lesion only.
4 = Lymphoid hyperplasia and exhaustion, increase of reticulo-endothelial cells.

of some muscle fibers, extreme atrophy of others, and rarely hyalinization gave microscopic support to the gross findings in most cases (Fig. 1). Five of the hearts were hypertrophied also, as judged at necropsy. Microscopic changes interpreted as compensatory hypertrophy were found to some degree in practically all of the hearts. These changes consisted of increased size or number of muscle nuclei, or both, sometimes accompanied by the presence of muscle fibers of unusually large diameter (Figs. 2 and 3).

In order to evaluate the accuracy of our judgment of the microscopic picture in these cases, both the size and the number of the nuclei in several hearts were determined by means of a micrometer eyepiece, and compared with hearts from the control pigs. The dimensions of the nuclei were measured directly, using the largest nuclei encountered in the myocardium of the animal in question and making no allowance for shrinkage which doubtless occurred in the preparation of the microscopic section. In determining the number of nuclei, all nuclei, whether in muscle fibers or in the endomysial connective tissue, were counted in an area which, for convenience, was 67 by 225 μ or, practically speaking, 15,000 sq. μ. An effort was made to use sections equal in thickness in all cases, as nearly as possible. No attempt was made to deduce means or averages lest a personal factor intervene through an unconscious selection of the fields to be counted.

TABLE IV
Analysis of Myocardial Hypertrophy

Animal number	5	7	10	Control	Control
Largest nuclei, μ					
Usual size	8 x 15	6 x 15	5 x 13	6 x 10	4 x 12
Exceptional size		10 x 25	6 x 18	7 x 11.5	
Nuclei, per 15,000 sq. μ					
Longitudinal section:					
Highest count	92	109	107	87	72
Lowest count	69	100	95	67	42
Cross section:					
Highest count	114	125	93	84	61
Lowest count	78	83	86	58	48

The results are given in Table IV and amply support the original microscopic impressions.

All of the livers were congested grossly, with at least a suspicion of intralobular necrosis or other degenerative changes. Generally speaking, the livers were reddish with the lobular architecture possibly

more prominent than normal. (In the pig this is normally conspicuous because of the distinct interlobular septa.) Microscopic examination revealed a startling change in all of the livers. While in 5 pigs the destruction was only partial, the remaining 13 had almost no viable parenchymal cells. In these only a narrow rim of hepatic cells remained at the extreme periphery of each lobule (Fig. 5). The remainder of the lobular space was filled with blood (Fig. 5), although a scattered reticulum of Kupffer cells appeared to be intact. While many poisons cause central or more extensive necrosis of the liver, usually with some degree of cloudy swelling or fatty change, I am not acquainted with any other intoxication which presents this extreme destruction.

In addition to the congestion and edema to which allusion has already been made, the lungs frequently displayed a very noticeable infiltration of the alveoli with macrophages of the well known "epithelioid" appearance. These, however, did not contain hemosiderin as might have been expected from the congested state of the lungs. Instead, their presence close to the alveolar walls, their greater frequency in peribronchial regions, and the co-existence of a slight thickening of the alveolar walls suggested that they were probably due to terminal infection. There were areas of genuine red hepatization in 5 animals, but these were never extensive and can be attributed to infection of previously devitalized pulmonary tissue. The pulmonary hemorrhages recorded in Table III were multiple in the 2 pigs affected but each involved less than 2 cc. of tissue.

In addition to congestion, the red pulp of the spleens of 10 animals was notably atrophic and deficient in lymphoid cells, possibly because of congestive anoxia. In several, however, the reticulo-endothelial and fibrous tissue was increased. The splenic nodules (malpighian corpuscles) suffered much less. Two spleens were considered edematous and in 2 the walls of arterioles appeared thickened. Atrophy of the lymphoid tissue in the congested and edematous lymph nodes was observed in 6 animals. Contrary to what was seen in the spleen, the follicles of the lymph nodes were not spared.

The renal lipidosis indicated in Table III was either in the proximal convoluted tubules or in the ascending loops of Henle, varying in different cases.

While extensive areas of the skeletal muscles frequently were pale or almost white, it was difficult to demonstrate microscopically any change beyond an abnormal variation in the size of certain fibers, some being atrophied, others hypertrophied (Fig. 4). Reproductive

and endocrine organs and the central nervous system revealed nothing of importance although they shared to some extent in the generalized congestion and edema.

Discussion

The lesions portrayed above are nearly identical to the lesions described by West[4] in dogs fed poisonous amounts of cottonseed meal except that gastro-enteritis was more prominent in the dogs and petechial and ecchymotic hemorrhages were numerous. The centrilobular destruction of liver cells was less pronounced in the dogs.

In contemplating the almost unprecedented changes found in the liver, the question arises whether this degree of hepatic injury results from the anoxia consequent upon congestion and stasis of blood or whether a direct hepatotoxic action must be attributed to the gossypol. Also advocated has been the theory that increased blood pressure in the intralobular capillaries destroys the hepatic cells by pressure necrosis. That severe chronic passive congestion is quite capable of bringing about centrilobular necrosis of this nature is generally accepted, and is supported by the work of such investigators as Lambert and Allison,[5] who studied 112 human cases of hepatic congestion in 1916. The loss of liver cells in such disorders as cardiac insufficiency and their replacement by overflow of blood from the capillary sinuses usually were limited to the central parts of the lobules. In comparing such changes with the almost complete involvement of lobules, seen in our cases, it presumably could be argued either that gossypol poisoning represents an exaggeration of what those authors found or that a different mechanism is involved. Those who hold that a direct toxic action from something imported by the blood is essential to cause such extensive death of hepatic cells have only to point to the multitude of poisons and toxic products of metabolism which are known to have this effect, usually without any notable alteration in the rate or character of the hepatic blood flow. Such morphologic evidence on this question as is afforded by the tissue sections themselves is equivocal. Against the concept of a direct toxic action upon the liver was the nearly complete absence of cloudy swelling or other toxic degenerative changes in the peripheral hepatic cells which survived. Fat stains made on the liver in certain pigs were uniformly negative. Possibly favoring a direct toxic action was the prevalence of cloudy swelling in the epithelial cells of the renal tubules. Narrowing of the hepatic cell cords which remained was observed in several of these livers, a feature upon which earlier pathologists relied to support the theory of pressure necrosis but which is probably

directly incident to the developing necrosis and not the result of pressure.

For purposes of comparison, as well as from the standpoint of practical differential diagnosis, three other disorders of animals producing a similar picture of hepatic destruction may well be considered. The first of these, poisoning by mouldy corn, has been studied especially by Sippel *et al.*[6] While widespread hemorrhages, as well as an entirely different clinical history, readily distinguish that condition from gossypol poisoning, the changes in the liver appear in some instances to be almost identical. The second comparable disorder is poisoning by the coal-tar pitch of the "clay pigeons" used as targets by trapshooters. Pigs sometimes get this material from the ground where the shattered targets have fallen and develop an acute poisoning characterized by a spectacular centrilobular necrosis and filling of the central part of the lobule with blood. However, the hepatic damage probably never involves the whole lobule; destruction of about half of it, as measured along the radius, is usual. No cardiac insufficiency has been noted in either of these poisonings.

The third comparable disorder is of dietetic nature and is the most intriguing, as well as the most perplexing. Hove and Seibold[7] fed a diet low in protein (soybean meal), markedly deficient in vitamin E and containing cod-liver oil, of which highly unsaturated fatty acids are characteristic constituents. After from 1 to 4 months on this diet the animals usually died and, at necropsy, showed a "hemorrhagic necrosis" of the liver similar in most respects to the hepatic condition ascribed to gossypol. In pigs that lived for 6 months, cirrhosis developed. The authors considered the livers to be comparable to those produced by Obel,[8] which will be described presently. Their pigs showed numerous hemorrhages in various organs, including lymph nodes, endocardium, epiglottis, lungs, and gastro-intestinal mucosa. They mentioned the absence of lesions in the skeletal muscles. Other organs apparently failed to attract attention.

Obel[8] described a porcine disease known as hepatosis diaetetica which occurs naturally in Sweden and Northern Europe and which was susceptible of experimental reproduction by the combined deleterious effects of (1) inadequate dietary protein (brewer's yeast, which is deficient in the sulfur-containing amino acids), (2) deficiency of vitamin E, and (3) presence in the diet of considerable amounts (6 per cent) of cod-liver oil with its highly unsaturated fatty acids. The similarities between the lesions arising from excessive gossypol and those described for natural or experimental cases of hepatosis diae-

tetica are seen not only in the necrotic and blood-filled hepatic lobules but also in the concomitant presence of extensive edema in practically identical situations, and in the presence of retrograde changes in skeletal and cardiac musculature which, while called by different names, appear microscopically to have at least the degenerative features in common.

On the other hand, certain notable differences between the cases of gossypol poisoning and those which Obel[8] and Hove and Seibold[7] found to result from dietetic imbalances are readily apparent both in clinical and post-mortem aspects. While Obel's pigs were reported as dying "quickly without the owner noticing any illness" (although occasional dyspnea is mentioned in exceptional cases), the gossypol-poisoned pigs suffered, usually for several days, from respiratory and cardiac difficulties which no one could have overlooked. In the liver, typically affected lobules were very similar in both conditions, all but the most peripheral hepatic cells being replaced by blood, but the distribution of such lobules was very patchy in the "deficiency" pigs, while in our "gossypol" pigs it was almost universal. The result was livers of entirely different gross appearance, spotted and patchy in the "deficiency" animals but evenly discolored in the "gossypol" pigs. Gastric ulceration, hemorrhages in various organs and tissues, and degenerative and necrotizing changes in arteries ("fibrinoid degeneration," "periarteritis nodosa") were accorded considerable prominence in Obel's descriptions but were absent or negligible in the gossypol syndrome. Obel's pigs sometimes developed the condition of yellow fat and ceroid pigment which has assumed prominence in connection with deficiency of vitamin E in company with an excess of unsaturated fatty acids in the diets of mink, as well as swine,[9] and which is well known in experimental rats. Changes of this sort were not a part of the gossypol picture.

In trying to understand the relationship, if any, among these several destroyers of hepatic cells we find that very similar hepatic changes result from such widely varying disorders as (1) stasis of the hepatic blood flow, (2) poisoning by products of the growth of moulds in decomposing corn, (3) poisoning by coal-tar pitch, a mixture of complex hydrocarbons, (4) poisoning by gossypol (and to a lesser degree by numerous other substances), and (5) by a diet unusually rich in unsaturated fats and at the same time deficient in vitamin E and certain proteins, probably those providing the sulfur-containing amino acids. Is there any common factor? For the first of the series, local anoxia seems an obvious result of failure of replenishment by means

of new blood. With respect to the dietetic causes, the chemistry is still obscure. Vitamin E is frequently called an "anti-oxidant" although little is said as to just what oxidations are inhibited. Unsaturated fats or fatty acids are substances that are easily oxidized. (Note their action in "drying oils.") Of the chemical mechanisms by which the several poisons exert their toxic effects, nothing is really known. It is doubtless an over-simplification to suggest that, in the absence of the moderating "anti-oxidant" action of vitamin E, the easily oxidizable fats of cod-liver or other fish oils rob the hepatic cells of an adequate amount of oxygen, but there is a strong probability that all these hepatotoxic disorders cause similar hepatic changes because they institute similar types of interference with local oxidative processes, with or without the added factor of inadequate blood supply.

Summary

The principal lesions of gossypol poisoning are congestion and edema resulting from myocardial injury. The edema is especially prominent in the lungs and body cavities. The congestion is of great severity in the liver, where it is accompanied by destruction of all but the most peripheral parts of the hepatic cords. This striking change in the liver is comparable to what is seen in two other poisonings, in anoxia of circulatory origin and in dietetic imbalance involving an excess of unsaturated fatty acids coupled with deficiency of vitamin E and of certain proteins. Interference with oxidative processes may possibly be the fundamental mechanism in all cases.

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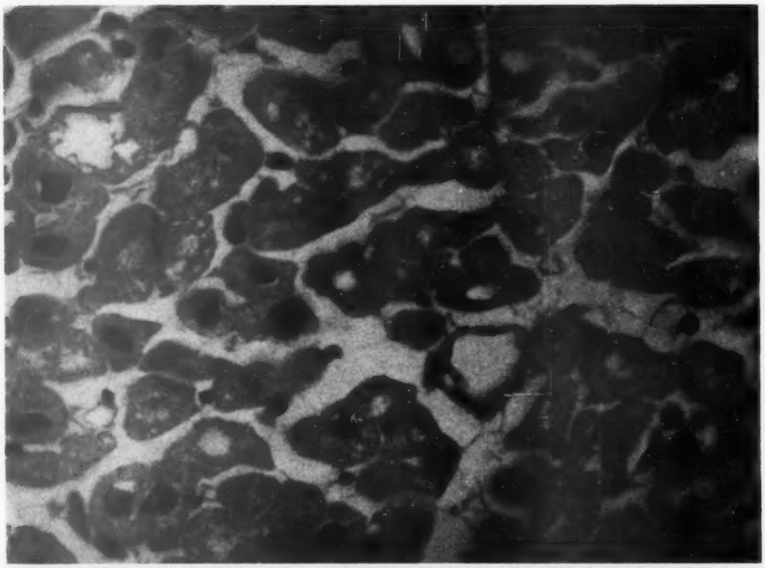

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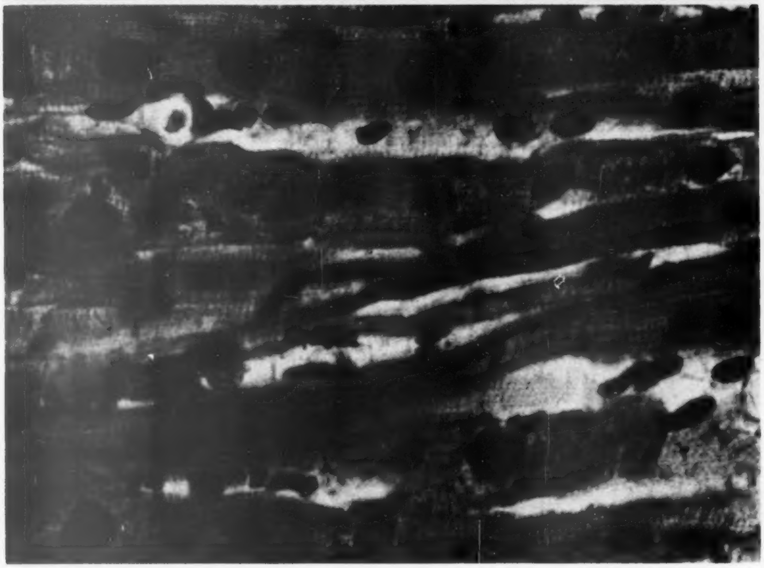


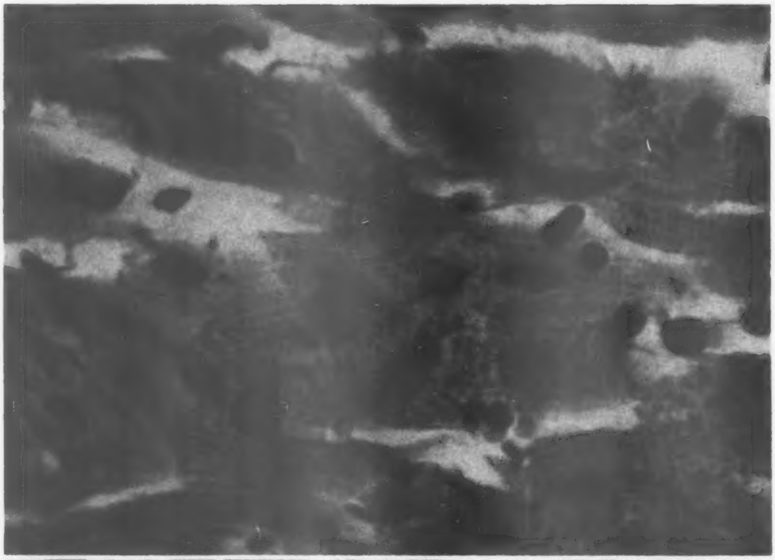
2

LEGENDS FOR FIGURES

FIG. 1. Vacuolization and other degenerative changes in myocardial fibers. Hematoxylin and eosin stain. × 400.

FIG. 2. Increased number of nuclei in myocardium. Hematoxylin and eosin stain. × 400.

3



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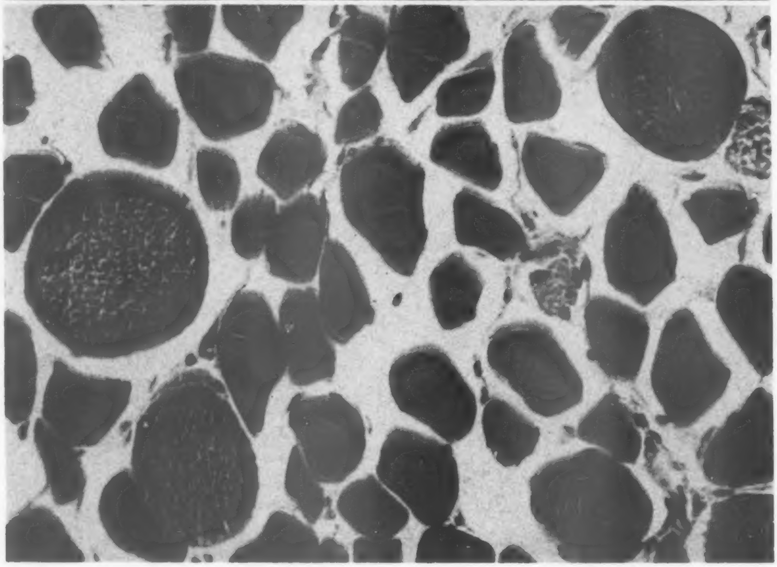

FIG. 3. Hypertrophic nucleus in midst of degenerative myocardium. Hematoxylin and eosin stain. × 400.

FIG. 4. Degenerative changes in skeletal muscle. Hematoxylin and eosin stain. × 600.

FIG. 5. Sector of a hepatic lobule, filled with blood except near the interlobular septum. Hematoxylin and eosin stain. × 175.

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